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**“ENDOTHELIAL DYSFUNCTION IN CHILDREN WITH CHRONIC RENAL
FAILURE: CAUSES AND THERAPIES”**

By

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MBBS MRCP**

Thesis

Submitted for the degree of

DOCTOR OF MEDICINE

of the

UNIVERSITY of LONDON

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For my husband Phillip and my son Ralph

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Publications arising from this work

“Oral L-arginine does not improve endothelial dysfunction in children with chronic renal failure”

Bennett-Richards K Katternhorn M Donald A Oakley G Varghese Z Deanfield J Rees L
Kidney International 2002; 62:1372-1378

“Does oral folic acid lower total homocysteine levels and improve endothelial function in children with chronic renal failure?”

Bennett-Richards K Katternhorn M Donald A Oakley G Varghese Z Rees L Deanfield J
Circulation 2002 Apr 16; 105(15):1810-5

Contributions made to this thesis

Dr Katy Bennett-Richards designed the studies, recruited the subjects and conducted the trials.

Endothelial function studies were performed mainly by Mia Katternhorn in addition to Ann Donald with the assistance of Dr Katy Bennett-Richards. Dr Bennett-Richards also reviewed the video-tapes, calculated the flows and analysed the data.

The blood samples were all drawn and prepared by Dr Katy Bennett-Richards. Studies of NO metabolism were carried out by Professor Bruckdorfer (Royal Free Hospital, London), LDL oxidative stress by Dr Varghese (Royal Free Hospital, London) and routine biochemistry, analogues of L-arginine and homocysteine by Mr. Michael Beauchamp on behalf of Dr Tony Reynolds (Great Ormond St Hospital, London).

Abstract

Premature death from cardiovascular disease is common amongst adult patients with chronic renal failure (CRF). The implication for paediatric patients, the majority of whom now survive into adulthood, is that they may be similarly affected but at an earlier age. Children with CRF already show signs of arterial disease in the absence of classical risk factors. The aim of this research was to develop ways of improving the outcome for children with CRF by investigating the effect of interventions on vascular function.

Vascular endothelial function, which is dependent on nitric oxide (NO) (an antiatherogenic substance) can be measured using a non-invasive technique of high resolution ultrasound. Abnormal responses represent the earliest change in the pathogenesis of vascular disease and may be reversible. Bioavailability of NO may be reduced in CRF due to low levels of precursors, increased levels of inhibitors and increased oxidant stresses.

The hypotheses examined were:

- 1) Dietary supplementation with L-arginine (the substrate for NO synthetase) improves large vessel endothelium-dependent vasodilatation and NO bioavailability.
- 2) Dietary supplementation with folic acid reduces homocysteine levels and improves large vessel endothelium-dependent dilatation.
- 3) Haemodialysis improves large vessel endothelium-dependent dilatation by lowering homocysteine levels, removing inhibitors of NO synthesis, reducing oxidative stress and/or removing free radicals.

Methodology was double blinded, randomized and placebo controlled in children with stable CRF in the absence of classical risk factors for atherosclerosis. Endothelial function was measured in the brachial artery. Biochemical measures of NO activity and oxidant stress were also measured, along with the acute effect of haemodialysis on endothelial function.

We demonstrated that L-arginine was not beneficial as a therapeutic agent. Folic acid improved vascular function and is now used routinely in all children with CRF at Gt Ormond St Hospital. Haemodialysis had an adverse effect on vascular function-an observation that needs further study as the process in itself may contribute to the development of atherosclerosis.

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Chapter one

Introduction

1.1 Epidemiology

1.1.1 Cardiovascular mortality, the size of the problem

Premature cardiovascular disease amongst adult renal failure patients represents a major cause of mortality and morbidity, some 10 to 40 times that of the general population. Nowhere is this more striking than in the younger age group.

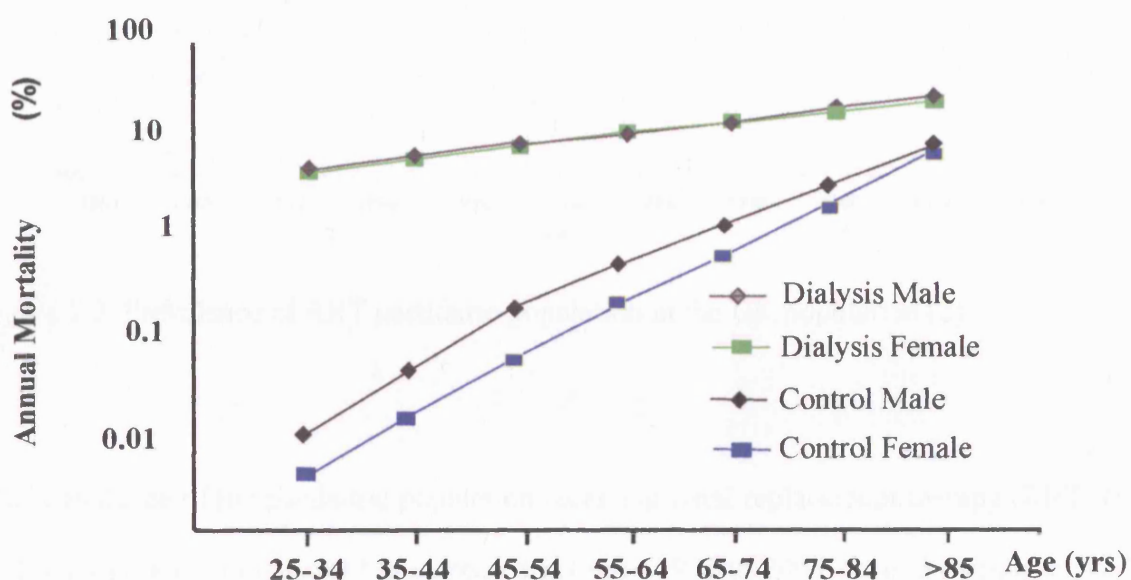


Figure 1.1: All-cause cardiovascular mortality for men and women in the general population and in individuals receiving haemodialysis (1)

This is of increasing concern in the paediatric renal failure population (considered to be <16 years) as most are now surviving into adulthood, both because of advances in dialysis and transplantation and because treatment is being extended to the youngest patients.

1.1.2 Prevalence

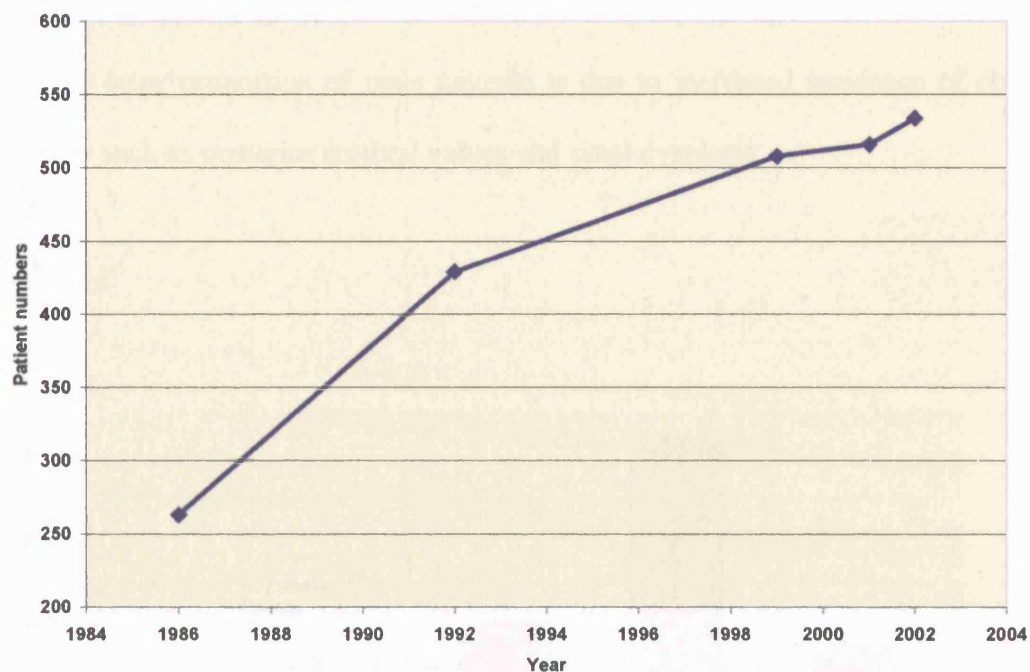


Figure 1.2: Prevalence of RRT paediatric population in the UK population (2)

The prevalence of the paediatric population receiving renal replacement therapy (RRT) (ie dialysis and renal transplant) has increased between 1992 to 2002, from 38.8 pmp to 52.4 pmp (2). The better survival of patients taken on at a younger age and increasing take on rates are responsible.

The distribution of ages for 2002 are shown in figure 1.3. The rising numbers in each age group reflects the survival of younger patients and comparatively higher take on rates in the 12 to 15 years age group. The fall off in the older age group is due to transfer to adult units. The large proportion of male patients is due to increased incidence of obstructive uropathies such as posterior urethral valves and renal dysplasia.

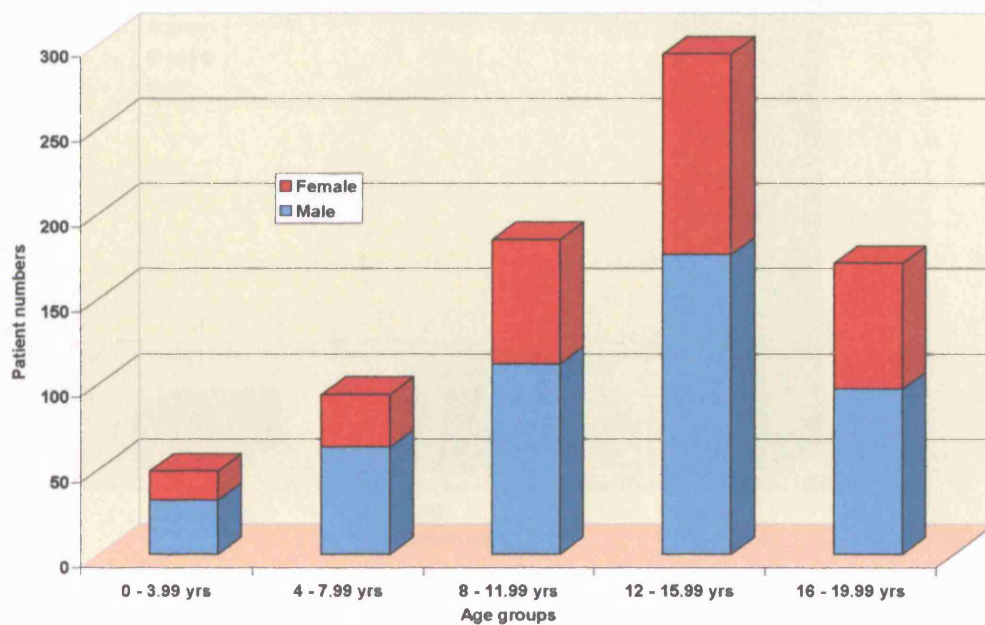


Figure 1.3: Age and sex distribution of the UK RRT in paediatric renal units in 2002 (2)

1.1.3 RRT modality

Renal replacement therapy consists of haemodialysis (HD), continuous ambulatory and cycling peritoneal dialysis (CAPD and CCPD), and renal transplantation both cadaveric (Cad) and live related (LRD). The distribution of these modalities for 2002 is as shown in figure 1.4.

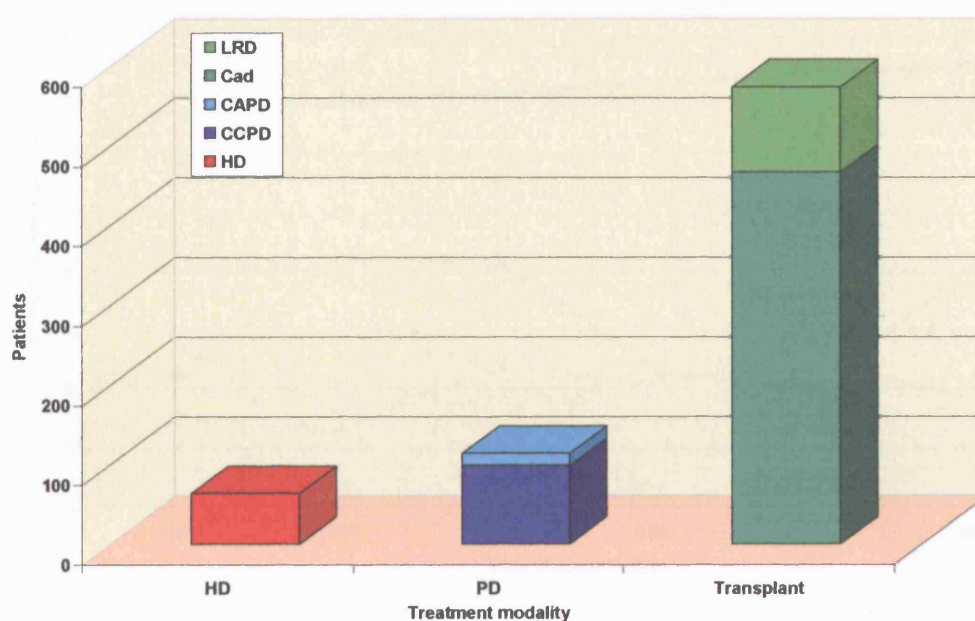


Figure 1.4: Current modality of RRT in paediatric units in April 2002 (2)

1.1.4 Take on rates of patients on to RRT programmes (incidence of end stage renal failure)

Take on rates have changed relatively little but there is a general upward trend also contributing to the size of the population.

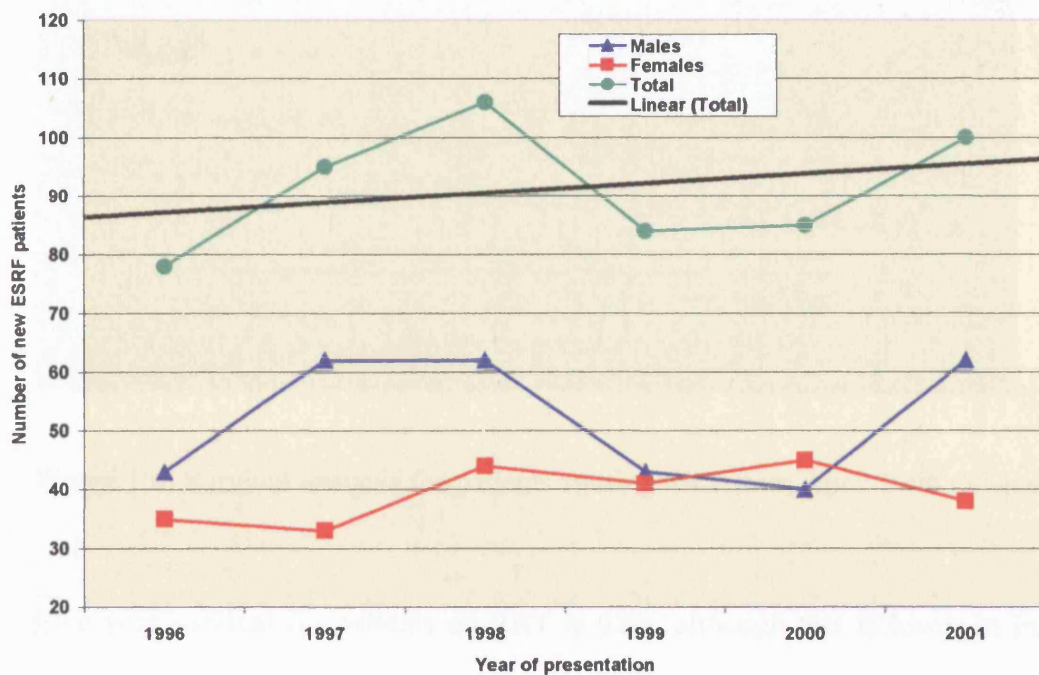


Figure1.5: New patients starting RRT in paediatric units in the UK from 1996 to 2002 (2)

1.1.5 Survival

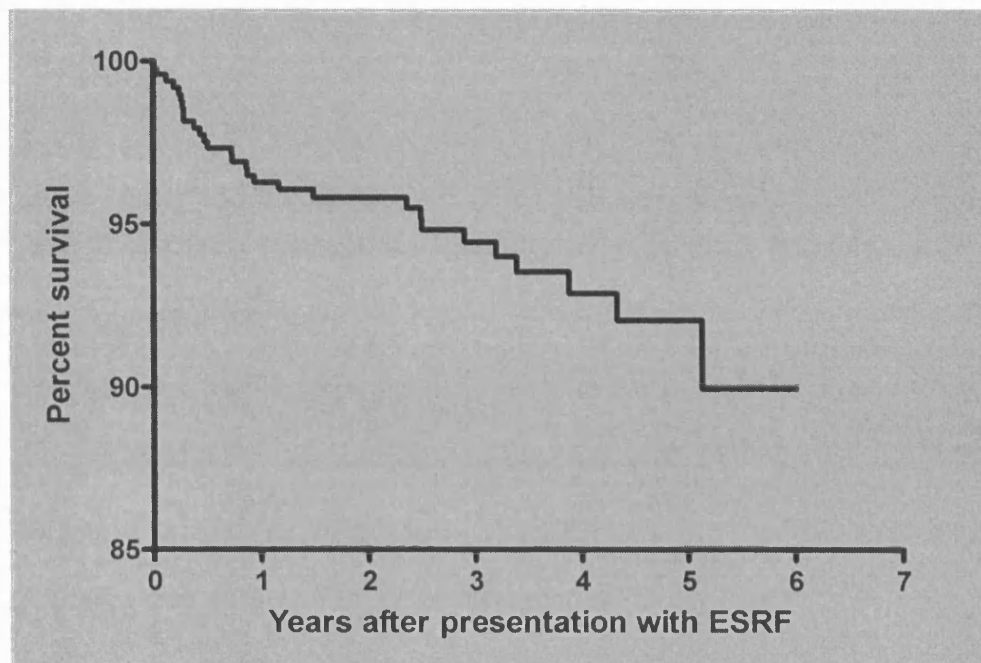


Figure 1.6: Survival analysis for patients starting RRT from April 1996 to March 2001 (2).

Five year survival for patients on RRT is 92%, although this is lower in infants (66%). Deaths are mostly due to dialysis access failure, infection and co-morbid conditions (usually in association with other congenital anomalies). With this generally good outcome we can only expect the size of the paediatric RRT group to increase further.

The long term mortality and morbidity in this group of renal failure patients is uncertain but they may be expected to have similar vascular complications as the adults but at an even earlier age. It has been demonstrated that children with chronic renal failure already show signs of early vascular damage (3). This is in the absence of classical risk factors for atherosclerosis such as hypertension, chronic fluid overload, and abnormal lipid profiles thus suggesting a “uraemic” factor responsible for the phenomenon.

1.2 Occurrence and pattern of cardiovascular death in CRF

1.2.1 Introduction

Death from cardiovascular related illness in renal failure is endemic and far exceeds that of matched population. Increased rates of 10 to 40 times have been reported. This pattern has not changed between 1974 and 1990, despite advances in RRT, and a known association between renal failure and premature death from cardiovascular disease (4,5).

This is of increasing concern with a reported growth of the adult end stage renal disease (ESRD) group of up to 70% in the last decade (6).

Nowhere is the increased risk of death from cardiovascular disease more striking than in the young haemodialysis population. Patients less than 35 years are 500 times more likely to die from vascular causes than their healthy peers (1). Although the incidence of established renal failure amongst children is fairly constant there has been a significant increase in the prevalence over the last 10 years. With more infants being offered dialysis and improved survival of children on RRT it is extremely likely that the effects of prolonged exposure to uraemic toxins will be associated with an increase in atherosclerotic morbidity and mortality similar to that seen in the adult population but occurring at an earlier age.

1.2.2 Adults

Studies in adults, both retrospective and prospective, have consistently demonstrated increased rates of cardiovascular mortality in renal failure patients (1, 5-10). Post mortem studies show that the presence of atherosclerotic disease in the conduit arteries of haemodialysis patients far exceeds that seen in non-uraemic individuals (8, 11). Death rates from myocardial infarction (MI) are reported at five times that of a control population and have an increased associated mortality (7). Peripheral vascular disease and cerebrovascular also more common (5, 12, 13).

This phenomenon of increased cardiovascular mortality was first noted in 1974 in haemodialysis patients and it was initially concluded by Lindner that the dialysis process itself was to blame (4). However it is thought that the atherosclerotic process begins much earlier. Indeed even a serum creatinine at $116\mu\text{mol/l}$ was found to be associated with increased risk of stroke and $130\mu\text{mol/l}$ with MI in a large prospective study. This is even after correction for traditional cardiovascular risk factors (14). This has been confirmed in a 10 year survey in adults with more significant CRF (creatinine clearance $30\text{-}35\text{ mls/min/1.73m}^2$) where a three fold risk of both MI and stroke were demonstrated (12). In the elderly (>65 years) a linear relationship was confirmed between glomerular filtration rate (GFR) and the incidence of cardiovascular events (15).

The reasons for these repeatedly reported increased rates in adults is thought to be related to two main factors: firstly the demographic changes in the dialysis population, ie increased age and primary aetiologies of renal failure ie diabetes; secondly the increased

prevalence of risk factors in the CRF population as compared with the general population. These were identified by the Framingham Study in the non-uraemic population as hypertension, lipid disturbances, left ventricular hypertrophy and glucose intolerance (16). In a prospective study examining comparative mortality from cardiovascular disease hypertension was seen in 63%, hypercholesterolaemia in 9%, cardiomegaly in 33% and diabetes in 13% of patients entering a dialysis programme. In addition 34% were smokers and 12% had suffered a previous MI or angina (10). Also, Culleton identified that mild CRF (serum creatinine 136-265 μ mol/l) was not only common in the general population, but was associated with a high prevalence of co-existing cardiovascular disease (17).

It is clear that a lot of the burden of CVD is related to the presence of risk factors but what about an additional role of uraemia itself and how early does the process begin?

1.2.3 Children

The understanding that the process of atherosclerosis begins early in life was probably first realized in 1953 from post mortem studies among soldiers (whose average age was 22 years) killed in action in North Korea. In 77.3% of cases some gross evidence of coronary atherosclerosis was found (18). This is a rather shocking statistic in what might be expected to be a relatively fit group of men. Further postmortem studies have shown that the initial fatty streaks, an early feature of atherosclerosis may be found after the first few months of life and increase in prevalence with age (19, 20).

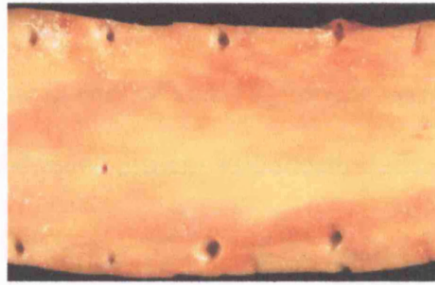


Figure 1.7: Fatty streaks in the aorta

Further evidence that the process has begun long before adulthood and may in fact be preprogrammed is that low birth weight is associated with impaired endothelial function (a process in the development of atherosclerosis) in the first decade of life, even before major exposure to classical risk factors (21). These abnormalities persist into adult life (22). It is therefore thought that another process is at work unrelated to the traditional risk factors.

With the development of non-invasive techniques the findings of post mortem studies can be further investigated in vivo, looking for evidence of atherosclerosis and identifying risk factors both classical and non classical, for example, obesity and type 1 diabetes in children is associated with arterial abnormalities in vivo (23, 24).

Prospective population studies in children have identified predictors of atherosclerosis in later life. Low density lipoprotein-c (LDL-c) levels and body mass index (BMI) in the Bogalusa Heart Study (486 children) (25), LDL-c, systolic BP, BMI and cigarette smoking in the Young Finns study (2229 children) (26) were identified as predictors using measurement of carotid intima-media thickness (a marker of preclinical atherosclerosis).

Less traditional risk factors have been identified in similar studies in children. These include elevated homocysteine in children with hypertension and fibrinogen levels in obese children (27) and an adipose derived protein adiponectin (28).

With regard renal failure populations evidence exists that the process maybe “accelerated” in both CRF and dialysis patients. Histological evidence was rather dramatically demonstrated by Nayir et al when they examined internal iliac artery segments of 12 children (11-17yrs) obtained at the time of renal transplantation. Five showed fibrous intimal thickening, two had micro calcification of the intima and two already had atheromatous plaques (29).

In vivo evidence has been demonstrated with impaired endothelial function seen in paediatric renal transplant patients (30) and perhaps what is of most relevance, in children with CRF and no classical risk factors (3). Endothelial studies in young adults (20-40years) with childhood onset CRF have confirmed the presence of subclinical atherosclerotic disease (31).

1.3 Arterial disease in CRF

1.3.1 Plaque morphology

There has been a concept of “accelerated atherosclerosis” initially observed in haemodialysis patients in 1974 to account for the increased mortality amongst renal failure patients. This was attributed to the process of haemodialysis rather than renal failure itself but some work has shown there is no relationship between time on haemodialysis and

increased risk (32) and that the vascular abnormalities predate initiation of dialysis (9, 32-34).

In 1979 it was noted that the large elastic arteries had marked intimal hyperplasia and striking medial calcification (11). This has been supported by further post mortem studies in adults which have shown heavy calcification in plaques (without any increase in the frequency of distribution) as compared to the mostly fibroatheromatous type seen in non-uraemic individuals (35). This may explain why an “accelerated” process is involved as these plaques behave differently as exemplified by high reocclusion rate within one year of 70% in uraemic patients compared to 40% in diabetics and 20% in non diabetics following percutaneous coronary angioplasty (36).

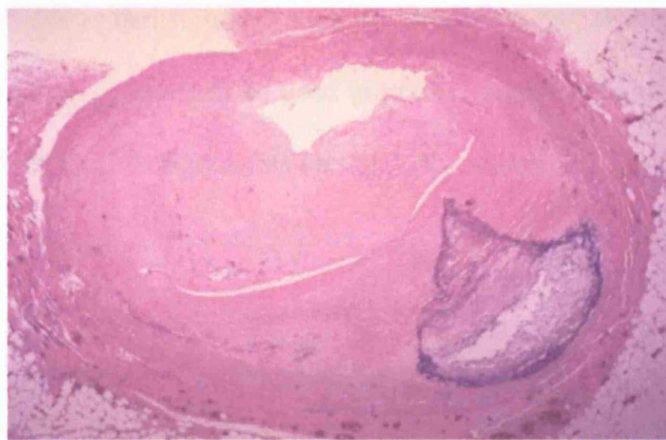


Figure 1.8: Histology of coronary arterial calcified atheromatous plaque in uraemia. Note increased intima media thickness and calcified areas (appear blue).

These observations of increased media thickness and calcification have formed the foundation of many of the non-invasive investigations used in both adults and children with CRF.

Electron-beam computerized tomography can determine the presence of arterial calcification in the coronary arteries that correlates well with the extent and severity of angiographic lesions (see paragraph 1.5.3.2). Haemodialysis patients aged 20-30 years have been shown to have significant coronary artery calcification compared with controls and younger dialysis patients (37). We know however that it does exist in younger patients as demonstrated in vivo in the internal iliac arteries by Nayir et al with a mean age of 15 years (29).

Intima-media thickness as measured by B mode ultrasound has also been used as a tool to monitor vascular pathology and can be used to predict cardiovascular mortality. Increased values have been seen in both predialysis and dialysis patients.

1.4 Endothelial physiology and pathophysiology

1.4.1 Normal abnormal and pathophysiology

It has become evident that the vascular endothelium, the single cell layer lining all blood vessels, is crucial in the control of vascular physiology. It has been recognised to be one of the body's largest and most complex organs with an overall weight similar to the liver. The endothelium separates the vessel wall from circulating components of blood and plays a

pivotal role in the defence against atherogenesis.

The endothelium regulates vascular tone, vascular smooth muscle cell proliferation, inflammation, permeability, cell adhesiveness and coagulation. It responds to a number of stimuli including neurotransmitters released from autonomic and sensory nerves (acetylcholine, norepinephrine, ATP, substance P), circulating hormones (catecholamines, vasopressin, angiotensin II, insulin) and locally active autoids produced both by endothelial and smooth muscle cells (bradykinin, adenine nucleotides and nucleosides, angiotensin and endothelin). In addition, the endothelium responds to physical stimuli, including changes in shear stress, which elicits flow-mediated vasodilatation (FMD).

1.4.1.1 Vascular tone

In response to these various stimuli, endothelial cells produce a variety of vasoactive substances, both constricting and dilating. Nitric oxide (NO) was identified initially as an endothelial relaxing factor (EDRF) following the observation of vasodilatation in isolated arteries after infusion of acetylcholine (38). Six years later this EDRF was discovered to be the freely diffusible gas, NO (39). It is continually produced in the endothelial cells by nitric oxide synthetase (eNOS), which incorporates molecular oxygen into the substrate L-arginine producing NO.

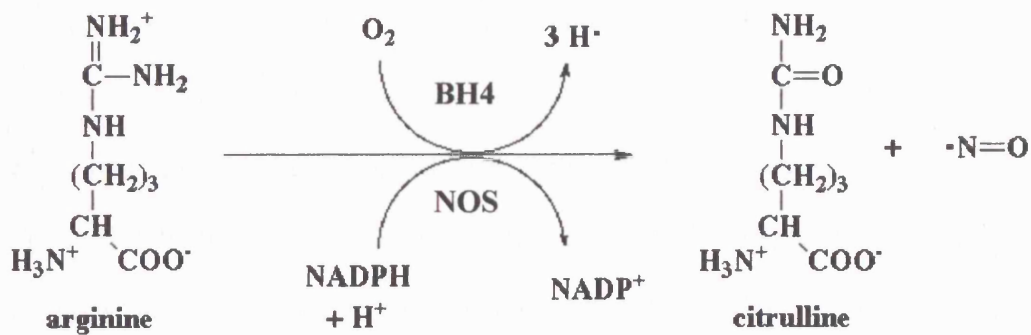


Figure 1.9: Conversion of L-arginine to NO and citrulline by NOS with BH4 as co-factor

NO diffuses readily through cells to act in the vascular compartment and on nearby vascular smooth muscle cells where it activates guanylate cyclase and increases production of cyclic 3', 5'guanosine monophosphate (cGMP). cGMP in turn reduces intra-cellular calcium within smooth muscle cells, and results in smooth muscle relaxation.

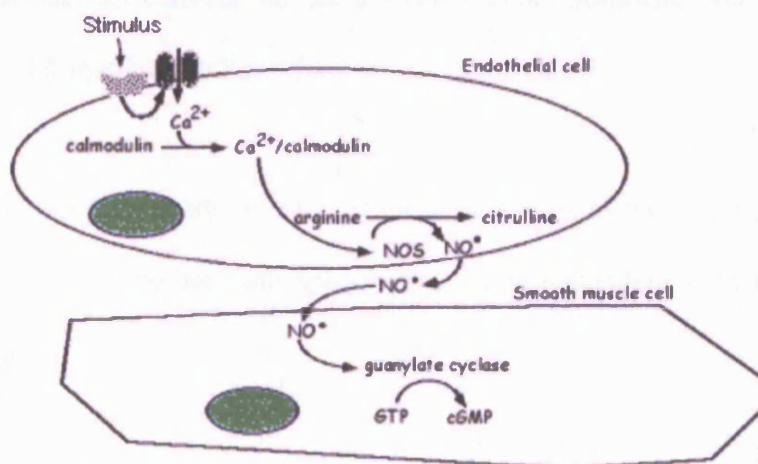


Figure 1.10 Endothelial cell NO production

Endothelial NOS binds to distinct domains of the plasma membrane called caveolae, which

are tethered to the cytoskeleton. Increased intracellular calcium facilitates calmodulin formation, which displaces the caveolin to activate the enzyme. This reaction is dependent on the presence of tetrahydrobiopterin, BH₄. Stimuli that increase intracellular calcium levels include receptor-mediated agonists, such as acetylcholine, bradykinin, substance P and the physical stimulus of endothelial cell shear stress. Thus these stimuli result in vasodilatation.

Other vasodilator substances produced by the endothelium are arachidonic acid derivatives (prostaglandins) and endothelium derived hyperpolarising factor (EDHF).

The effects of these relaxing factors are continually counterbalanced to varying degrees by vasoconstricting factors. These are thromboxane A₂, superoxide anion, angiotensin II, and endothelins. The endothelins are a family of peptides which mainly act on smooth muscle cells to bring about vasoconstriction but a small number stimulate NO and prostacyclin release thus providing a feedback mechanism.

The specific contributions made by each of these mediators to the endothelium-dependent regulation of vascular tone are both species and tissue dependent with the main players being NO and endothelin.

1.4.1.2 Smooth muscle cell proliferation

In the presence of healthy endothelium smooth muscle cell turnover remains static. This is due to a balance of growth promoters, angiotensin II, endothelin, and platelet derived growth factor with inhibitors such as heparin, NO and transforming factor- β (TGF β). If the endothelium is denuded under experimental conditions proliferation is the dominant

process producing a neo intima.

1.4.1.3 Control of permeability

The intact endothelium is able to control the passage of solutes, water and cells across its surface. Pores exist for water and solutes but the passage of larger molecules and proteins is controlled by a dynamic actin filament system at intercellular junctions. Some endothelial factors will increase permeability and others will reduce it. During the inflammatory process leucocytes will cross the barrier. This is controlled by adhesion molecules (intercellular adhesion molecules, ICAM and vascular cell adhesion molecules, VCAM) expressed on the surface of the endothelial cell.

1.4.1.4 Haemostasis

Endothelial cells both inhibit platelet aggregation via NO and prostacyclin and provide a non thrombogenic surface due to their ability to inactivate factor X and thrombin in addition to activating protein C through binding thrombin.

1.4.2 Endothelial function and atherosclerosis

An impaired or abnormal response of the endothelium is termed “endothelial dysfunction” and is generally characterised by impaired bioavailability of NO, the main participant in maintaining a healthy endothelium. Inhibition of NO production in experimental animal models causes accelerated atherosclerosis (40). Endothelial dysfunction has been widely shown to be the earliest clinically detectable pathological process leading to the development of atherosclerotic disease (41-43).

It has been repeatedly demonstrated that endothelial dysfunction exists in the form of abnormal endothelial dependent responses (to chemical or mechanical stimuli) in coronary, conduit and resistance vessels both in patients with established clinical atherosclerosis and in those at risk of atherosclerosis. These include hypertension diabetes, coronary artery disease, cigarette smoking, increasing age and hypercholesterolaemia (44-48).

Of importance it has been noted that the presence of endothelial dysfunction in conduit vessels correlates well with the presence of established atherosclerotic disease in the coronary circulation (49, 50).

It also is a predictor of subsequent development of clinically significant disease. In patients with mild coronary artery disease, the presence of coronary endothelial dysfunction (using acetylcholine) was associated with a major cardiac event over a 28 month follow up period (51). This finding was confirmed in another study with a 7.7 year follow up. It was also noted that coronary endothelial function was an independent predictor even after adjustment for traditional cardiovascular risks and established disease (52).

The prognostic value of endothelial dysfunction of the forearm conduit arteries (as oppose to coronary vessels) has been demonstrated in untreated hypertensive patients where impaired responses are a predictor of cardiovascular events including stroke and myocardial infarction (53).

These studies suggest that detecting endothelial function is important as it may allow intervention with therapeutic options before atherosclerosis is established.

1.4.3 Endothelial Dysfunction in CRF

Animal studies have demonstrated that experimentally induced uraemia can impair acetylcholine (Ach) induced vasodilatation ie bring about endothelial dysfunction (54, 55).

In adult humans endothelial dysfunction has been demonstrated using endothelial dependent stimuli such as Ach and sheer stress models in both chronic and endstage renal disease (both haemodialysis and peritoneal dialysis) on repeated occasions (32, 33, 56-58). In these patients many of the traditional risk factors associated with atherosclerosis are present such as smoking, diabetes and hypertension.

In addition endothelial dysfunction has been demonstrated also to have a predictive value when considering all cause mortality in end stage renal disease (59).

Studies in children with CRF might overcome this hurdle of confounding factors in search for a uraemic factor that may be responsible for the excess deaths from cardiovascular disease in this group. A study by Kari et al showed that in children with CRF who were not hypertensive, diabetic or smokers and had normal total cholesterol levels that endothelial function was impaired when compared with matched controls (3).

This may be due to reduced bioavailability of NO as a consequence of reduced availability of substrate, increased levels of inhibitors, and increased removal of NO.

1.5 Assessment of endothelial function and abnormal arterial pathophysiology

1.5.1 Introduction

The ability of the endothelium to respond normally can be tested in vitro or in vivo by using endothelial dependent stimuli and comparing them to endothelial independent stimuli. In vivo tests can also be subdivided into invasive and non-invasive. In addition the characteristic histopathological abnormalities of the arterial tree in renal failure has led to the development of other non-invasive techniques to assess extent of early subclinical cardiovascular disease.

1.5.2 In vitro

Isolated vessels such as mesenteric artery are placed in organ baths and mounted on myographs. Their response to acetylcholine (endothelial dependent dilator) and sodium nitroprusside (endothelial independent dilator, ie donor of NO) are recorded and compared. Animal experiments by Kakoki and Ruschitza demonstrated impaired endothelial function in the aortic and renal arteries using rat models of acute renal ischaemia (54, 55).

1.5.3 Invasive in vivo techniques

Invasive in vivo techniques include venous occlusion plethysmography, which is based on the observation that vasodilatation of the forearm microvasculature is associated with a proportional increase in blood flow into the forearm. An upper arm occluding BP cuff is inflated sufficiently to occlude venous return, but insufficient to impair arterial filling.

Arterial blood continues to enter, and forearm volume expands. The rate of arterial blood flow into the forearm is proportional to the rate of rise in forearm volume. Strain gauges are attached to the forearm to measure forearm blood flow from changes in forearm circumference. The effects of various pharmacological agents on blood flow can be examined in particular endothelial dependent dilator agents (acetylcholine, bradykinin) can be compared with endothelial independent agents such as glyceryl trinitrate (GTN) when looking for evidence of endothelial dysfunction. London et al used this technique to demonstrate an impaired response in haemodialysis patients, which was also found to be an independent predictor of all cause mortality over a 60 month follow up period (59).

Good correlation between strain gauge plethysmography and flow mediated dilatation of the brachial artery exists (60).

During coronary angiography the measurement of Doppler blood flow velocity in the coronary arteries in response to endothelial dependent stimuli such as acetylcholine, increased blood flow using papaverin and cold pressor testing, can be used to assess endothelial function. This technique was used to demonstrate that although the coronary arteries in patients with hypercholesterolaemia were angiographically normal endothelial dysfunction was present (42).

1.5.4 Non-invasive in vivo techniques

Invasive techniques are not practical when carrying out large scale investigations and studies on children. The phenomenon of endothelial function fortunately lends itself well

to assessment by non-invasive methods and these more recently have become the main stay of investigation into endothelial dysfunction, particularly in children and young adults.

1.5.4.1 Flow mediated dilatation

Flow mediated dilatation (FMD) uses a high resolution, external ultrasound assessment of conduit arterial vasculature, usually the brachial artery. It was developed in 1989 and is based on the observation that the stimulus of flow produces dilatation in the arteries (61).

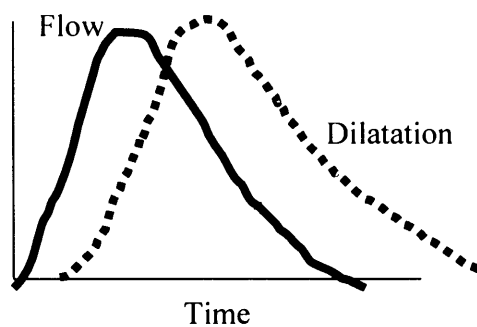


Figure 1.11: Relationship between blood flow and vessel dilatation

This process is endothelial dependent and was shown to be in part NO dependent (62, 63). Vascular activity in response to endothelial dependent stimuli, increased flow, is contrasted with an endothelial independent stimulus, systemic glyceryl trinitrate which is a direct donor of NO. Arterial diameter is measured at rest and following a brief period of reactive hyperaemia produced by suprasystolic inflation of a cuff distal to the site of measurement. The percentage change in brachial vessel size is recorded. This is then compared with the endothelial independent stimulus, GTN, given sublingually which assesses the integrity of the smooth muscle response. The two recordings are compared. Impaired responses using this technique have been found in some high risk adult groups such as, patients under 40 years with coronary artery disease (50), CRF (33), haemodialysis and peritoneal dialysis

patients (57, 58). In children, impaired endothelial dependent responses have been found in obesity and type 1 diabetes (23, 24, 64, 65). Of particular interest is the study by Kari et al who noted an impaired response in children with CRF of 4.9 % compared to 8.9% ($p=0.0001$) in matched controls where endothelial independent responses were similar (25.1 % v 23.3% ($p=0.31$)) (3). This has also been demonstrated in renal transplant children with stable graft function (29).

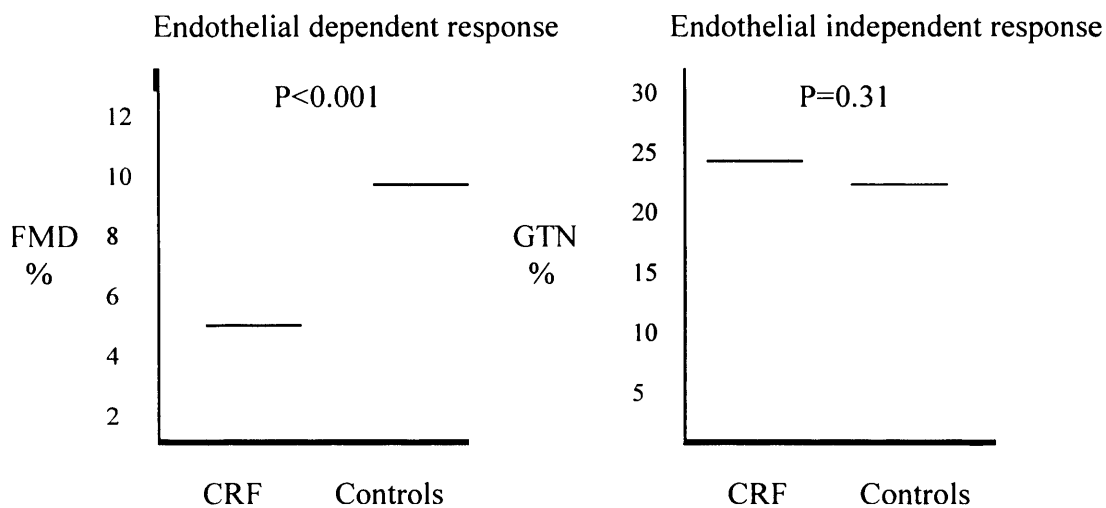


Figure 1.12: Impaired endothelial function demonstrated in children with CRF (3)

1.5.4.2 Other non invasive methods of assessing abnormal arterial pathophysiology

As discussed earlier the pathophysiology of atherosclerosis in renal failure is characterised by vascular calcification, which leads to structural and functional disturbances in the arterial tree. This manifests itself by reduced arterial compliance and arterial wall thickening. Techniques of pulse wave velocity (PWV), intima-media thickness (IMT) and electron beam computerised tomography (EBCT) have been employed as they detect such abnormalities and have been shown to be significant predictors of cardiovascular mortality (66).

Pulse wave velocity

Arterial stiffness is characteristic of atherosclerosis. Wada et al demonstrated a clear association between arterial stiffness (as measured using the relationship between vessel diameter and its change with blood pressure) and the presence of atherosclerotic lesions seen in the same subjects at postmortem (67). The technique of pulse wave velocity (PWV) has been developed as a more direct method of assessing arterial stiffness. This is the measurement of the velocity of the pulse wave when traveling between two sites of the arterial tree (usually common carotid and femoral arteries) using transducers connected to an automated on line calculating device. An increased value indicates increased stiffness of the arterial segment being observed. It has been demonstrated to be both reliable and repeatable. In addition, aortic PWV in hypertensive (both treated and untreated) individuals is not only strongly associated with the presence and extent of atherosclerotic disease (defined on the basis of clinical events) but it has also been demonstrated that even a single recorded value of $>13\text{m/s}$ is a strong predictor of cardiovascular mortality (68).

Arterial stiffness has also been demonstrated in ESRD population on haemodialysis (69). Shinohara found that the presence of predialysis renal failure was also significantly associated with increased aortic PWV, independent of age, gender, blood pressure, BMI, smoking, and adverse lipid profiles and that in fact haemodialysis had a favourable effect on arterial stiffness with reduced values seen when compared with predialysis patients (70). Even in young patients (<55 years) with mild to moderate renal failure a negative correlation exists between PWV and creatinine clearance independent of blood pressure and classical cardiovascular risk (71).

In addition to this, the predictive power of PWV is also applicable to the dialysis population. PWV, in addition to age and time on dialysis in a prospective trial was demonstrated to be better than pulse pressure or left ventricular mass at predicting cardiovascular mortality (72).

It has therefore been considered a valuable tool when carrying out large scale observational and interventional studies of the clinical effectiveness of therapies.

Intima-Media Thickness

Arterial IMT (carotid, femoral) is an index of thickening of the arterial wall.

Using B-mode high resolution ultrasound this can be measured reliably. It is an independent predictor of cardiovascular mortality in nonuraemic individuals after adjustment for traditional risk factors (66). In hypercholesterolaemic children studies have demonstrated increased carotid IMT compared to controls (73, 74). As autopsy changes suggest that the first atherosclerotic lesions develop in the abdominal aorta. Jarvisalo et al in 2001 studied the distal aortic IMT in addition to carotid IMT. They discovered that in addition to significantly greater IMT in the high risk children (hypercholesterolaemic and type 1 diabetics, n=60, aged 11 ± 2 yrs) than in the controls, there was a relatively larger difference in aortic IMT than carotid IMT in the high risk group. They suggested therefore that aIMT was the best non-invasive predictor of preclinical atherosclerosis in children (75).

Using this technique in the uraemic population increased carotid IMT is seen in both CRF predialysis patients 0.889 ± 0.035 versus 0.685 ± 0.01 mm in controls $p<0.001$ (34) and haemodialysis patients (76).

Electron Beam CT

High resolution transverse images of the heart are obtained and a cumulative calcification score is generated for all the coronary arteries.

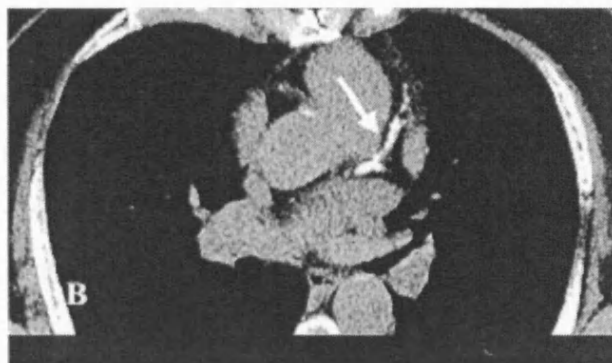


Figure 1.13: EBCT image showing calcification of left coronary artery

The extent of coronary artery calcification (CAC) as measured by EBCT correlates with the extent and severity of angiographically documented lesions in the non-uraemic population (77, 78, 79) and with previous myocardial infarction (80). This has been found to have a predictive value for coronary events in asymptomatic individuals but the relationship to clinically significant lesions remains controversial (81). Calcification was found in 88% of young patients (20-30 years) with CRF on dialysis and almost absent in matched controls (82). Medial calcification may be responsible as EBCT is not capable of distinguishing between intimal and medial calcification so the higher calcification scores may reflect the media wall changes rather than purely intimal changes. Haydar et al examined the relationship between CAC and coronary artery disease in the 46 uraemic patients. They discovered that as in the non-uraemic population there was a strong correlation between the extent of coronary artery disease as demonstrated by coronary angiography and CAC score as measured by EBCT. Further more by multivariate regression analysis they showed that CAC score was the only predictor of burden of coronary artery disease in their study population (83).

1.6 Risk factors for atherosclerosis in CRF

1.6.1 Introduction

Many adults with CRF also have the traditional risk factors for cardiovascular disease such as diabetes, hypertension, smoking, dyslipidaemia (10, 17). The purpose of this thesis is to examine the effect of CRF per se on the development of atherosclerosis. Here the key to endothelial dysfunction is thought to be impaired bioavailability of NO. This may be due to reduced levels of substrate, impaired production or increased inactivation. In addition factors relevant to CRF are high homocysteine levels, increase oxidant stress, abnormal calcium phosphate metabolism and anaemia.

1.6.2 L-arginine

The semi-essential amino acid L-arginine is the only substrate for NO synthesis. Serum levels of L-arginine have been reported as being low in CRF but this is not a universal finding. The intra-cellular concentrations of L-arginine far exceed the K_m value for NO synthase (NOS) making it unlikely that it is simply L-arginine concentrations which are the rate limiting step for NO production. It has been demonstrated that high but physiological levels of LDL may affect cellular uptake of L-arginine and hence NO production (84).

1.6.3 Accumulation of endogenous inhibitors of NO synthase

In CRF decreased clearance results in high levels of circulating analogues of L-arginine, asymmetrical dimethylarginine (ADMA) and its stereoisomer, symmetrical

dimethylarginine, SDMA (85). ADMA has been characterised as an endogenous inhibitor of NOS, the enzyme which catalyses the production of NO from L-arginine in vascular endothelial cells (86). ADMA acts by competitively displacing L-arginine from its binding site on NOS. This is even at concentrations seen in patients with CRF. ADMA levels have also been shown to have a predictive value when considering cardiovascular mortality in dialysis patients (87) and to be positively associated with carotid artery IMT in non-dialysis patients (88). Currently a prospective trial is looking at the prognostic value in an unselected group as elevated levels are also seen in patients with cardiovascular disease, hypertension, chronic heart failure and hypercholesterolaemia (89).

1.6.4 Hyperhomocysteinaemia

The observation in 1969 that patients with congenital defects of homocysteine metabolism resulting in hyperhomocysteinaemia (levels $>50\mu\text{mol/l}$ nr $5-15\mu\text{mol/l}$) die from premature vascular disease led to the investigation of homocysteine as causal in the pathogenesis of atherosclerosis. High levels have subsequently been noted in high risk populations including CRF in adults (90) where there is an inverse relationship to GFR (91). Elevated levels have also been noted in children with CRF and on dialysis (92-94).

One possible mechanism therefore for endothelial damage in CRF is the presence of high circulating levels of homocysteine. Homocysteine is a sulphur containing amino acid formed as an intermediate during the metabolism of methionine. It has been shown in population studies to be an independent risk factor for both vascular disease (95, 96) and myocardial infarction (97, 98). In CRF, homocysteine is also an independent risk factor

(99) and in dialysis patients, hyperhomocysteinaemia is more prevalent than traditional cardiovascular risk factors (100).

In vitro and in vivo studies suggest that homocysteine causes endothelial dysfunction either directly or via intermediate reactions increasing oxidised LDL levels (101) and hence increased rates of NO inactivation. This is supported by studies, which have shown the effects of acute hyperhomocysteinaemia on endothelial function can be reversed with vitamin C (102) and that NO dependent flow mediated dilatation is impaired in hyperhomocysteinaemia (103). It is thought also to have a directly toxic effect on the endothelium, to increase platelet aggregation and smooth muscle proliferation. Even modestly elevated homocysteine levels may be particularly damaging in the presence of the atherogenic risk profile of CRF (104).

1.6.5. Increased oxidant stress

Many cell types in vivo, continuously form reactive oxygen species, primarily as a result of energy production. These are a family of molecules, including molecular oxygen produced by all aerobic cells. At physiological concentrations they are necessary for normal cell function such as signaling and cell growth. They are mostly free radicals (ie have an unpaired electron) such as superoxide (O_2^-) and hydroxyl (HO^-) and include nitric oxide (NO). Other reactive oxygen species include hydrogen peroxide (H_2O_2) and peroxynitrite. These are not free radicals in that they do not possess unpaired electrons, but still have oxidizing effects.

In health there is a balance between the production of these molecules and counter activity of antioxidant systems. Overproduction of reactive oxidant species overwhelming

antioxidant defence is referred to as a state of increased oxidant stress and can occur in response to inflammation, infection, environmental pollutants and malignancy. The result of which is the oxidative damage of DNA, lipids, proteins and carbohydrates.

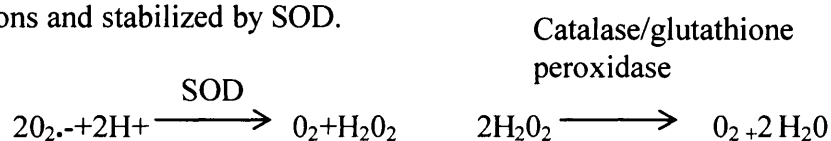
Oxidised lipids for example can be mutagenic and cytotoxic, particularly to the endothelium. In addition it has been demonstrated that oxidised lipids can affect endothelial NOS expression and hence NO production (105). Increased oxidant stress also produces activation of oxidant responsive genes leading to production of growth factors, chemokines and adhesion molecules that enhance the inflammatory response (106).

It is therefore no surprise that oxidant stress is thought to be a key factor in the adverse vascular biology seen in hypertension, diabetes, heart failure and hypercholesterolaemia.

Effects of reactive oxygen species
Reduced BH4 levels
Increased ADMA levels
Peroxidation of lipids
Inhibition of cGMP
Increased platelet aggregation
Increased expression of endothelial cell adhesion molecules
Increased expression of platelet derived growth factor on smooth muscle cells
Oxidative damage of DNA

Table 1.1: Summary of the effects of reactive oxygen species

Superoxide dismutase (SOD) is part of the intracellular antioxidant system enzyme found in both the cytoplasm and mitochondria, which mops up superoxide. NO can be inactivated by superoxide ions and stabilized by SOD.



As there is a much faster reaction rate between NO and superoxide than between SOD and superoxide there is always likely to be some inactivation of NO but under physiological conditions antioxidant defences will minimise it. In pathological situations of excess superoxide the reaction between superoxide ions and NO is the dominant pathway resulting in the formation of peroxynitrite. Peroxynitrite at low concentration has similar actions to NO but at high concentrations it is a potent cytotoxic agent causing protein fragmentation by nitration of amino acids such as tyrosine residues to form nitrotyrosine. These are found in excess in atherosclerotic plaques in humans.

The role of LDL

It has been shown that under conditions of increased, but still physiological concentrations of native LDL and oxidised LDL, that there is a conformational change in endothelial NOS (eNOS) which promotes generation of superoxide in preference to NO. This was partly inhibited by the addition of L-arginine and suggested that LDL affects uptake of L-arginine (84). Also Wever et al also observed that during hyperlipidaemia eNOS might contribute to oxidative stress by a reduction in tetrahydrobiopterin (BH4) dependent NO formation (107). These support several other studies which have shown that in the absence of L-arginine or tetrahydrobiopterin eNOS can produce superoxide and H₂O₂. This has been referred to as NOS uncoupling (108).

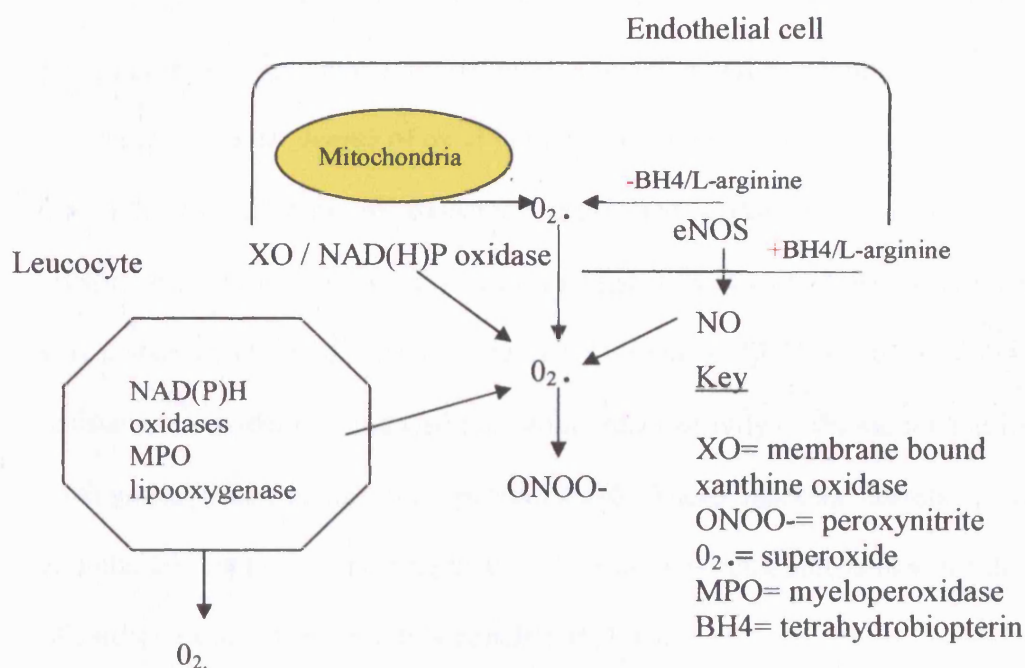


Figure 1.14: Pathways of increased oxidant stress

Oxidant stress and CRF

There is evidence that renal failure is a state of increased oxidant stress. This is through indirect evidence with raised levels of oxidised lipids, markers of lipid peroxidation (109, 110), lowered measured total antioxidant activity (111) and increased level of antibodies to oxidised LDL (112). Antibodies to oxidised LDL have been demonstrated to have an independent predictive value of cardiovascular mortality in dialysis patients (113).

Low levels of NO metabolites have been measured in the urine of CRF patients suggesting that NO production is down-regulated and that oxidative stress may be increased (114).

The presence of oxidant stress (using oxidatively modified amino acids and plasma proteins) has been shown to be present even at GFR of 60ml/min and showed no correlation with the degree of renal failure below this (115).

The relationship between oxidative stress and endothelial function in CRF (mean creatinine clearance 25mls/min) was investigated by Annuk et al who confirmed that CRF was a state of elevated oxidative stress with reduced LDL lag times (a measure of LDL resistance to oxidation), reduced total antioxidant activity of the serum and increased diene conjugates, (markers of lipid peroxidation). These markers correlated with impaired endothelial function suggesting that this was an important mechanism for the development of cardiovascular disease in this population (116).

1.6.6 Reduced antioxidant capacity

Antioxidant defences counterbalance the production of reactive oxygen species by preventing free radical formation, blocking chain reactions and repairing oxidative damage. They may be intra or extra cellular, proteins, enzymes or vitamins either obtained from the diet or naturally present within cells.

Glutathione (GSH) is the primary nonenzymatic cellular antioxidant system found in all cellular compartments. Vitamin E is a family of lipid soluble compounds (tocopherols) and resides in plasma lipoproteins and cell membranes where it is best placed to prevent lipid peroxidation. Vitamin C takes part in the regeneration of vitamin E by scavenging free radicals. Antioxidant enzyme systems consist of SOD, the absence of which is lethal,

catalase and glutathione peroxidase. These all convert free radicals to less reactive forms. For example glutathione peroxidase detoxifies hydrogen peroxide to water.

Extra cellular antioxidants consist of proteins such as transferrin, albumin, urate and ceruloplasmin.

Total antioxidant capacity has repeatedly been demonstrated as low in CRF. There is evidence that the activity of the enzymes SOD, catalase and glutathione peroxidase are reduced and that levels of antioxidant vitamins are low (116-120). Annuk showed that there were reduced levels of GSH (the most important cellular antioxidant) and increased levels of oxidised glutathione (GSSG) in renal failure compared to normal controls (116). Children with CRF (5-18 years) have been shown to have reduced SOD and glutathione peroxidase activity in addition to reduced levels of co-factors for these enzymes such as selenium and copper. The degree of antioxidant capacity was related to the severity of the CRF (121).

Some studies have shown acute benefit of antioxidant vitamin supplementation in states of endothelial dysfunction such renal failure (122).

1.6.7 Abnormal phosphate, calcium and parathyroid hormone (PTH) metabolism.

In renal failure disturbances of calcium and phosphate metabolism are common. These changes include raised plasma calcium and phosphate levels, secondary hyperparathyroidism, reduced synthesis of active vitamin D metabolites, reduced expression of calcium receptors and the use of vitamin D derivatives and calcium

supplements. Evidence suggests that these abnormalities may contribute to the development of vascular dysfunction and calcification (123). Cardiovascular morbidity and mortality correlates with abnormalities of calcium and phosphate metabolism. Hyperphosphataemia predicts reduced survival due to excess cardiovascular mortality in haemodialysis patients. Individuals in the highest quintile for plasma phosphate had a 52% greater risk of death from coronary artery disease, 39% greater risk of stroke, 34% greater risk of death from other cardiac causes and a 26% increased risk of sudden death compared to those in the lowest quintile (124). Vascular calcification as detected by EBCT in young adults (20-30 years) on dialysis was higher in those with greater calcium phosphate product (37).

The mechanism for this increased mortality in altered calcium phosphate metabolism is likely to be due to not only calcification of the arterial tree but also calcification of cardiac valves, myocardium and left ventricular hypertrophy (LVH). LVH is not only very common in renal failure, up to 41% in adult predialysis patients, (125) but has also been reported as an independent risk factor for cardiac mortality with two thirds of dialysis patients with LVH dying of heart failure or sudden death (126).

Major positive associations with the development of LVH have been hypertension and anaemia (127), which may be self-explanatory, but also PTH is thought play a leading role. PTH in vitro has both inotropic and chronotropic effects on cardiomyocytes (128). Of more interest perhaps is that cultured ventricular myocytes exposed to PTH increased protein synthesis indicating a trophic effect causing interstitial fibrosis (129). Parathyroidectomy has been shown to improve left ventricular function (130).

1.6.7 Anaemia

Anaemia has been reported as an independent risk factor for cardiac disease and mortality (131). A doubling of the risk of death has been seen in patients with Hb<8g/dl compared with those >11g/dl (132). However the use of recombinant erythropoietin has been effective in reducing mortality in haemodialysis patients (133) and producing regression of left ventricular hypertrophy in both dialysis and CRF patients (134).

Adverse effects of increasing the haematocrit using erythropoietin was noted in haemodialysis patients over 65 years with clinically evident cardiac disease by Besarab et al (135). This highlights the possible pro-hypertensive effect of erythropoietin.

1.6.8 Dyslipidaemia

Dyslipidaemia in CRF is characterised by an accumulation of triglyceride-rich VLDL, IDL and LDL remnants with reduced concentrations of cardioprotective HDL as a result of reduced tissue lipase activity. These abnormalities progress with renal impairment (136).

Cholesterol concentrations are similar to those seen in the healthy general population and may even be lower. This is often a reflection of malnutrition. Normal or low cholesterol concentrations often mask an abnormal lipid sub-fraction profile, which has an excess of pro-atherogenic small dense LDL particle. These are more susceptible to oxidation and have a higher cholesterol content (137). Oxidised LDL is toxic to the endothelium and can breach the intima. It can inhibit endothelial dependent vasodilatation and induce apoptosis of endothelial cells. This abnormal triglyceride/cholesterol ratio is due to reduced activity

of the enzymes which control lipoprotein degradation.

Levels of Lipoprotein (a), a large apoprotein associated with LDL particles are raised in early renal impairment as a result of excess production. They are pro-atherogenic and are an important predictor of cardiovascular morbidity in the general population (138). Lp(a) accumulates in the vascular wall at sites of atheroma formation and is also prone to oxidation

1.6.9 Inflammation

Inflammation has been identified as an important process in the initiation and progression of atherosclerosis. The earliest lesion, the fatty streak is a pure inflammatory lesion consisting of monocyte derived macrophages and T- lymphocytes. This process is thought to be initiated by some sort of toxic injury such as oxidised-LDL, which could affect NO production resulting in endothelial dysfunction. The inflammatory response via cytokines, chemokines and growth factors stimulates the migration of and proliferation of smooth muscle to form an intermediate lesion. Eventually through repeated accumulation of mononuclear cells and the formation of fibrous tissue an advanced complicated lesion is formed consisting of a fibrous cap with a core of lipid and necrotic tissue.

Acute phase proteins are proteins that change in concentration by at least 25% in inflammatory disorders.

CRP is an acute phase protein the concentration of which alters by 1000 fold in inflammation and has been identified as a predictor of cardiovascular mortality in non-uraemic populations (139). Elevated CRP levels have been found in dialysis patients in the absence of infection (140) and in predialysis patients even those with a GFR of up to

59mls/min (141, 115). More importantly these elevated levels have been found repeatedly to be an independent predictor of all cause morbidity in CRF (142-146).

1.7 Theoretical therapies

1.7.1 L-arginine

It is widely accepted that the earliest changes in the development of atherosclerosis are at the level of the endothelium. The intact healthy endothelium produces NO which is the main player in antiatherogenesis (Section 1.4). It is therefore conceivable that by increasing NO production the process of atherosclerosis may be attenuated or even reversed. As L-arginine is the only substrate for NO production it could be hypothesized that by supplementing with L-arginine, NO production could be enhanced. Kanno et al in 1992 showed that L-arginine increased urinary metabolites of NO and cGMP which would support this theory (147).

There are several ways in which supplementation with L-arginine might influence the bioavailability of NO particularly in CRF: it may correct substrate deficiency the kidney is the main source of L-arginine (148), overcome competitive antagonism due to ADMA, and increase production to overcome rapid removal due to increased oxidative stress. Experimentally in animals L-arginine has been used to inhibit atherogenesis in hypercholesterolaemic rabbits by inhibiting platelet activation (149) and improve endothelial dependent vasodilatation, firstly by Cooke in 1992 (150) and subsequently by Jeremy in 1996 (151).

Dietary L-arginine supplementation in humans has already been shown to improve endothelial function in the brachial artery of young people with hypercholesterolaemia (152); reduce the adhesiveness of mononuclear cells in hypercholesterolaemic humans (153); improve endothelial responses associated with raised ADMA (154); improve coronary small vessel endothelial function and symptoms (155) and improve symptoms in patients with heart failure (156).

In the setting of animal studies of CRF (the 5/6 nephrectomised rat) L-arginine has been shown to ameliorate glomerulosclerosis although the mechanism was initially not examined (157). Subsequently it was shown by Ashab et al that L-arginine had not only a beneficial effect on CRF by improving creatinine clearance and reducing proteinuria but that it also restored NO production which was initially lower than in controls (158).

In renal failure studies in adults results have been variable. Hand used an acute infusion of L-arginine to restore predialysis endothelial function to the better postdialysis values suggesting that haemodialysis improves endothelial function by increasing NO bioavailability (159). Cross et al found no benefit of acute L-arginine on chronically impaired endothelial function in haemodialysis patients (160). De Nicola et al assessed the effect of oral L-arginine on NO production in CRF and found no benefit, measuring urinary cGMP levels (161). Prior to my studies L-arginine had not been used as a therapeutic tool in renal failure patients but had been used subsequently in children with chronic kidney allograft dysfunction on cyclosporin to assess its effect on proteinuria and renal function (162).

L-arginine is thought also to mediate beneficial effects on vascular biology as it is capable of reducing total homocysteine levels by increasing NO production. NO converts homocysteine to an antioxidant and vasodilatory compound, S-nitrohomocysteine (163).

1.7.2 Folic acid

Homocysteine levels are an independent predictor of cardiovascular mortality both in the general population and in CRF. Folic acid has been shown to lower homocysteine in non-uraemic individuals (164) and uraemic adults (90, 165) and children (94), by increasing tissue methylation of homocysteine to form methionine. This is true even in the presence of normal folate level. Homocysteine can also be metabolized to cysteine as part of the transulfuration pathway, which requires the co-factor vitamin B6.

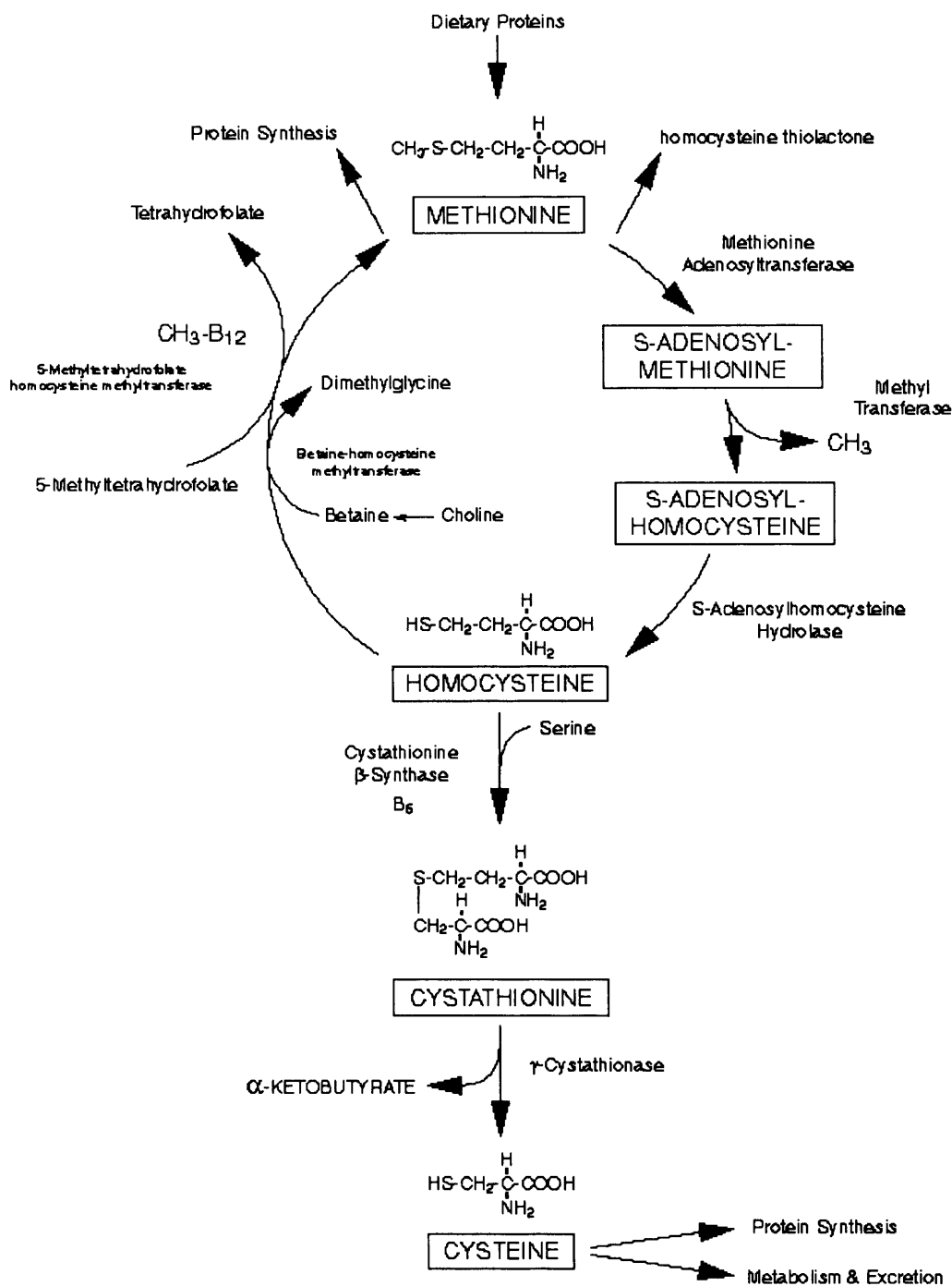


Figure 1.15: Homocysteine methionine pathway (the sulphur containing amino acid pathway).

It may be postulated that by lowering homocysteine levels a beneficial effect on the development of cardiovascular disease could be seen.

In studies in adults with hyperhomocysteinaemia and familial hypercholesterolaemia folic acid supplementation has resulted in not only a lowering of homocysteine but improvement in endothelial function (166-168). In 36 children (mean age 13.6yrs) with type 1 diabetes, 5mg folic acid improved FMD by 2.58% (169). This was independent of total homocysteine levels which were not elevated at the start of the study, suggesting an additional direct benefit on endothelial function.

Folic acid levels (or indeed B12) are not reported to be low in CRF, even in children and there is no correlation between the vitamin levels and homocysteine levels. Infact levels of folic acid were high in one study, possibly due to impaired clearance as they were highest in the dialysis patients (170).

In CRF it appears therefore that there maybe a relative resistance to folic acid or that serum levels do not reflect intracellular levels.

The active metabolites of folic acid such as methyltetrahydrofolate (MTHF) have been more successful at lowering homocysteine in CRF dialysis patients (171). However this is not a consistent finding. A similar dose was given to a larger group of 50 dialysis patients and showed no superiority over folic acid (172). The same group had shown earlier no extra benefit from supplementing with B12 or B6, essential co-factors (173).

In addition to the above mechanism folates are postulated to increase concentrations of tetrahydrobiopterin, which is an essential co-factor in the production of NO. This was thought to be the mechanism of improvement in endothelial function seen in hypercholesterolaemics as a fall in superoxide production was noted in vitro (174).

The impact on endothelial function in adults with CRF, on haemodialysis or peritoneal dialysis has however been disappointing (175,176,177). This maybe due to the likelihood of established vascular disease, the presence of multiple risk factors and because supplementation rarely reduces homocysteine levels to within the normal range (175).

1.7.3 Anti-oxidants

Antioxidant vitamins, by scavenging free radicals could theoretically increase NO bioavailability by protecting NO from inactivation by free radicals.

Animal studies have demonstrated that anti-oxidants can improve endothelial dependent dilatation (178) and this effect has also been observed in human clinical studies.

1.7.3.1 Vitamin C

Vitamin C is the primary water-soluble antioxidant in human plasma and early epidemiological studies showed that Vitamin C levels correlated inversely with hypertension and cardiovascular disease (179). It has also been demonstrated that 500mg of Vitamin C can significantly lower blood pressure in hypertensives (180). It may therefore follow that it could improve vascular biology in the setting of endothelial dysfunction. Vitamin C, when given acutely to hypercholesterolaemic individuals (181) and patients with coronary artery disease (182) produced a beneficial effect on endothelial-dependent function not seen on endothelial independent function or normal volunteers. More sustained, chronic administration (4 weeks) of Vitamin C in hypertensive individuals by Duffy et al showed no benefit on endothelial function in adults with hypertension (183).

In children with familial hypercholesterolaemia chronic administration of vitamin C and E improved FMD (184).

In the renal setting, vitamin C was first used in renal transplant patients. Endothelial dysfunction was demonstrated to be independent of uraemia, hypertension and treatment with cyclosporine (185). Williams et al (186) used 2g of vitamin C given acutely to improve endothelial function. This was associated with an increased resistance of the serum to oxidation, thus supporting the idea that the beneficial effect was through antioxidant capacity and that the pre-existing adverse biology may be due to increased oxidative stress. This effect has been transferred to the haemodialysis population where benefit was seen in the resistance vessels but not conduit vessels and was mediated by increased NO bioavailability (187).

This beneficial effect is thought to be due to free radical scavenging playing a significant role in regulation of intracellular redox states (188), prevention of oxidation of LDL (189) and increasing prostacyclin concentration (190).

1.7.3.2 Vitamin E and beta-carotene

Other antioxidant vitamins include vitamin E and Beta-carotene. These are lipid soluble. Vitamin E inhibits lipid peroxidation of LDL by scavenging lipid peroxide radicals and the carotenes by scavenging peroxy radicals. As oxidative modification of lipids is key for the development of atherosclerosis, defects in this system may predispose to adverse vascular biology.

Animal studies have shown that supplementation with vitamin E in hypercholesterolaemia can attenuate the development of atherosclerosis (191, 192). Epidemiological studies by

Rimm, Stampfer and Kushi examining the relationship between heart disease and vitamin intake according to diet and supplements suggest that vitamin E may be cardioprotective (193-195). Prospective clinical intervention studies in man have been less consistent, some have shown benefit (196, 197) where others have not (198) but Vitamin E given for 4 weeks to 7 subjects with hypercholesterolaemia did show a beneficial effect on NO production (199).

Levels of lipophyllic antioxidants have been found to be low in haemodialysis patients (200) and Vitamin E coated dialysers used in haemodialysis reduce oxidative stress and reversed the adverse effect of non biocompatible dialyser on endothelial function (201). A prospective study (SPACE) by Boaz et al demonstrated that Vitamin E reduced composite CVD and MI in adults on haemodialysis (202).

1.7.4 Statins

Clinical studies have shown that the use of statins (HMG-CoA reductase inhibitors) to lower cholesterol reduces the risk of cardiovascular events (203, 204). In addition to the direct effect of lipid lowering these drugs are thought to have a beneficial effect on endothelial function (205). Indeed this effect has been demonstrated in patients with coronary artery disease (206), normocholesterolaemic healthy volunteers (207) and post-menopausal women both without any change in lipid profile (208). This effect was also found to be sustained in an 18 month study of FMD in patients on statin therapy (209).

The mechanism of this effect may be by potentiated NO production as in the studies by Mercurio et al the effect was blunted by L-NMMA, an inhibitor of NO production (208). Statins are also thought to confer benefit by suppressing inflammation. Dalla Nora et al

showed that statins lower markers of leucocyte adhesion in type II diabetics (210). Other potential mechanisms are a direct antioxidant effect (demonstrated in vitro), an antithrombogenic effect, and inhibition of both smooth muscle proliferation and uptake of LDL into monocytes leading to the formation of the foam cell. The major culprit in the formation of the atherosclerotic plaque (211).

As chronic renal failure patients have adverse lipid profiles (high Tg, LDL, IDL reduced HDL, but with normal or low cholesterol, which separates them from the aforementioned groups) and are in a state of chronic inflammation with increased oxidative stress, statins could potentially be used to improve vascular morbidity and endothelial function. They have been shown to improve lipid profiles in CRF (212) and more importantly result in long-term reductions in cardiovascular mortality and all cause mortality (213). Benefits in endothelial function as measured by FMD have been seen in renal transplant patients with hypercholesterolaemia (214).

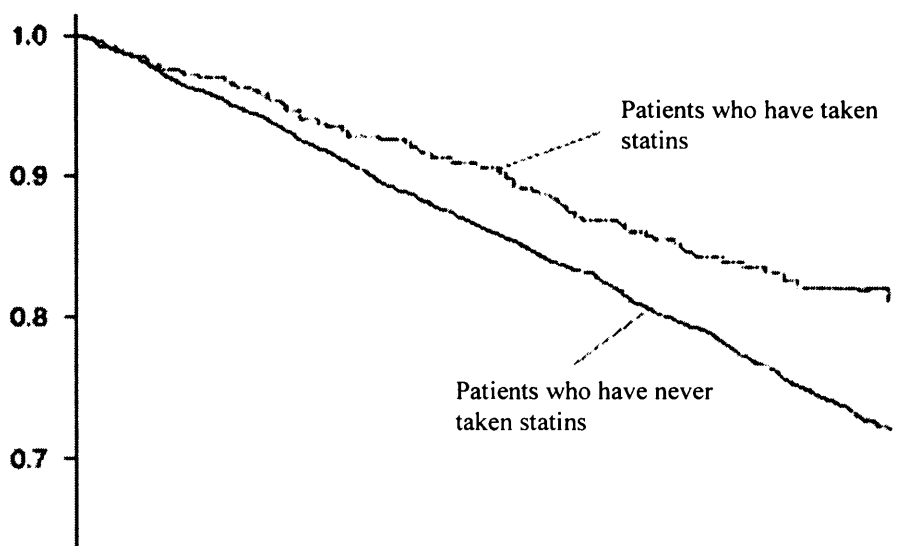


Figure 1.16: Adjusted Kaplan-Meier survival curves for all cause mortality in haemodialysis patients (from 213)

On going prospective studies (CHORUS) in haemodialysis patients are examining the potential mechanisms of vascular damage by measuring inflammatory proteins (ICAM, IL-6, monocyte chemoattractant protein) and markers of cardiac muscle pathology (troponin I and T) in addition to cardiovascular morbidity and mortality (215).

The use of statins in children was originally associated with concerns about adverse effects on growth. When simvastatin at 40mg was used in 173 children with familial hypercholesterolaemia, no adverse effect on growth or pubertal development was demonstrated over a 28 week follow up (216). A further study in 50 children (nine to eighteen years) showed in addition to beneficial effects on lipid profiles, improvement in endothelial function as measured by FMD (217). Their use in children with CRF has not been investigated.

1.7.5 Other theoretical therapies

Other theoretical therapies include plant sterols. These have been used safely to lower LDL levels but did not improve endothelial function in children (5-12yrs) with familial hypercholesterolaemia (218).

ACE inhibitors in adults on haemodialysis have been shown to increase serological markers of antioxidant defences (219). ACE inhibitors are not licensed for use in children but could potentially improve endothelial dysfunction.

Erythropoietin is a glycoprotein hormone produced by the kidney that promotes proliferation and differentiation of erythrocyte precursors. A recombinant form has been

developed and is in regular use to treat the anaemia associated with CRF. Overall treatment is beneficial but a major adverse side effect is hypertension and in investigating the mechanism for this it has been discovered that it is capable, in vitro, of up-regulating NO production (two to four fold) by increasing inducible NOS transcription in the normal endothelial cells in culture (220). However, in aortic segments from rats with CRF no such benefit was seen and those treated with erythropoietin had an augmented hypertensive response (221). It may be however therefore that some of the beneficial effects are mediated by its potential to overcome increased oxidant stress provided its use is not accompanied by hypertension.

Tetrahydrobiopterin is a necessary co-factor for the production of NO by endothelial NOS. It can be given orally and has been shown to improve endothelial function in adults with hypercholesterolaemia (222). Studies in acutely induced ischaemic renal failure in animals showed that impaired endothelial function could be restored by oral tetrahydrobiopterin when given both before and after the injury (54). This suggests it may potentially be of benefit in endothelial dysfunction in humans with CRF.

1.8 Nature of the studies

It has already been demonstrated that the process of atherosclerosis begins in childhood in the presence of CRF. Impaired endothelial function, the earliest detectable change in the process of atherosclerosis, was seen in the absence of classical risk factors. This has two main implications. Firstly that atherosclerosis is likely to develop early in this group, which is expanding; and secondly that this may be the period when the process is reversible and therapies should be considered.

Studies in this thesis examined possible therapeutic interventions and investigated them in children with stable chronic renal failure. In addition the possibility that in the end stage renal disease population, the process of haemodialysis itself was atherogenic was examined.

1.9 Hypotheses

The original hypothesis are therefore

1.9.1 Dietary supplementation with L-arginine (the substrate for NO synthetase) improves large vessel endothelium-dependent vasodilatation and NO bioavailability.

1.9.2 Dietary supplementation with folic acid reduces homocysteine levels and improves large vessel endothelium-dependent dilatation.

1.9.3 Haemodialysis improves large vessel endothelium-dependent dilatation by lowering homocysteine levels, removing inhibitors of NO synthesis, reducing oxidative stress and/or removing free radicals.

Chapter Two

The subjects

2.1 Introduction

Children with CRF and those requiring dialysis provide an ideal model to study the pathogenesis of atherosclerosis in renal failure. In all adult studies confounding variables make interpretation of the results difficult in addition to the fact that the presence of established vascular disease limits validity of the study of therapeutic interventions. In children not only does the absence of hypertension, diabetes, ischaemic heart disease and peripheral vascular disease provide a pure model of renal failure, such that the “uraemic factor” can be studied but also vascular disease is generally not yet established making therapeutic intervention studies more valuable.

This chapter provides a summary of the clinical and epidemiological features of the study population.

2.2 L-arginine and folic acid study subjects

2.2.1 Selection

Subjects were recruited prospectively from the general nephrology clinics held weekly at Great Ormond St Hospital. Children were aged from 7 years in order that they could co-operate with the study. Informed consent was obtained from the parents or guardians following an interview in which details of the study were explained and written information was given.

Inclusion criteria were children with stable CRF and an absence of the classical risk factors associated with the development of cardiovascular disease (hypertension, diabetes,

smoking, obesity and hypercholesterolaemia). Children with nephritic syndrome and those who were taking any vasoactive medication which may interfere with the study were excluded. Of the 25 recruited for the L-arginine study, 12 of them took part in the folic acid study in addition to 13 new subjects.

We did not preselect children on the basis of endothelial function in order that the study group should be representative of all children with moderate to severe CRF.

2.2.2 Characteristics

Thirty seven children (20 boys and 17 girls), mean age was 12 ± 3 years (range 7-19 years) with stable CRF were studied.

Subject	Age (yrs)	Sex	Wt (kgs)	Height Cms	GFR (mls/min)	Serum creatinine (μmol/L)	Aetiology of CRF
L-arginine study							
1 RS	14	M	59.5	156.0	25	214	Reflux and dysplasia
2 CL	14	F	48.0	160.0	34	160	Dysfunctional bladder
3 FH	13	F	35.0	143.0	10	538	Single kidney, megaureter
5 SK	13	M	35.0	133.0	38	170	PU Valves
6 MM	14	M	50.0	170.0	48	130	PU Valves
7 JR	10	M	34.0	134.0	34	165	Reflux
8 MW	14	M	34.1	150.5	10	500	PU valves Single kidney
9 BC	13	M	34.0	144.0	15	384	Dysplasia
10 CJ	14	M	51.5	152.5	41	117	Dysplasia and reflux
11 BW	9	M	22.0	121.5	19	276	PU valves
12 AS	13	M	47.0	148.5	34	105	PU valves
13 MJ	14	F	63.0	164.1	30	147	Reflux
14 JB	13	M	47.0	152.4	34	105	PU valves
15 KV	12	F	43.0	134.5	40	108	Dysplasia
16 TP	15	M	47.7	170.3	48	99	Dysplasia
17 SS	19	M	64.5	173.0	9	747	Dysplasia
18 ZI	10	M	20.5	116.0	46	78	Cystic renal disease
19 CS	7	F	16.4	105.0	9	445	Cortical necrosis
20 SH	7	F	22.1	120.0	25	180	Unknown
21a GC	7	F	19.0	114.0	29	102	Single kidney with Pyelonephritis
21b AB	9	F	20.3	118.0	19	101	Dysplasia
22 DM	15	F	52.0	170.0	14	500	Reflux
23 ZG	11	M	36.0	146.5	48	103	PU valves
24 AC	15	F	60.9	160.0	10	650	Single Kidney with PUJ obstruction
25 KW	7	F	35.0	132.0	32	128	AR polycystic kidney disease

Table 2.1A: Characteristics of study subjects (L-arginine)

Subject	Age (years)	Sex	Weight (Kgs)	Height (cms)	GFR (mls/min)	Creatinine ($\mu\text{mol/L}$)	Aetiology of CRF
Folic acid study							
1 CH	11	F	22.2	120	10	233	Cystic renal disease
2 HJ	8	F	21.5	123.5	40	93	Renal vein thrombosis
4 EC	15	F	58.0	152.0	15	369	Dysplasia
8 KJ	14	F	58.5	155.6	41	109	Dysplasia
10 RF	8	M	20.5	120.0	35	92	Dysplasia
13 HW	12	M	24.5	135.0	19	219	Cortical necrosis
14 JN	9	M	25.5	122.5	16	292	Dysplasia
15 MR	9	M	31.5	126.8	17	448	Dysplasia
16 JH	11	M	38.0	140.0	24	234	Reflux
21 LS	10	F	37.9	145.3	34	132	Dysplasia
23 JT	16	M	52.0	163.2	35	182	Dysplasia
25 ZQ	8	F	19.0	N/A	20	143	Unknown

Table 2.1B: Characteristics of study subjects (folic acid)

2.3 Haemodialysis study subjects

2.3.1 Selection

Seven stable children receiving euloaemic haemodialysis at Great Ormond St Hospital were entered into the study. Exclusion criteria were age <7 years (to ensure cooperation with the study), CRF of inflammatory cause, diabetes, arterio-venous fistulae in both arms, significant arterial/venous stenoses in proximal blood vessels, concurrent medication with immunosuppressants, and those with inter-dialytic fluid gains.

2.3.2 Characteristics

Seven children (3 boys and 4 girls, mean age was 12 ± 3 years (range 8-17 years) established on haemodialysis were studied.

Subject	Age (yrs)	Sex	Wt (kgs)	Aetiology of ESRF	Drugs	Access	Dialyser
1	13	M	42	Dysplasia	Frusemide Nifedipine Atenolol Ca carbonate Onealpha	Permcath	COBE500
2	8	F	36	Reflux	Na bicarbonate Na Cl Ca carbonate Erythropoietin	Permcath	COBE400
3	9	F	37.5	Congenital Nephrotic	Onealpha Ca carbonate Erythropoietin	Permcath	NIPRO FP70
4	17	F	52.7	Reflux	Nifedipine Atenolol Ca carbonate Onealpha Erythropoietin	Permcath	COBE500
5	9	F	33.7	Dysplasia	Ca carbonate	Permcath	COBE500
6	13	M	42.2	Wilms/Drash	Ca carbonate Erythropoietin Folic acid Onealpha	AVF	COBE500
7	15	M	80.5	Dysplasia	Onealpha Na bicarbonate Ca carbonate	Permcath	COBE500

Table 2.2: Characteristics of haemodialysis study subjects

Chapter Three

Common Methodology

3.1 Introduction

Elements of the studies which are common to all will be discussed here. This therefore includes a description of assessment of endothelial function using the technique of flow mediated dilatation (FMD), biochemical assays and statistics. The local ethics committee approved all the studies and informed consent was obtained from parents or guardians.

3.2. Measurement of endothelial function

3.2.1 Technique

Endothelial function was determined by recording the dilator response of the brachial artery to increase blood flow generated during reactive hyperaemia of the downstream forearm, flow mediated dilatation. Subjects lay supine in a temperature-controlled laboratory (22-25°C). The brachial artery was scanned in longitudinal section using a 7 MHz linear array transducer and an XP 128/10 (Acuson), magnified using a resolution box function and gated with the R wave of the ECG.

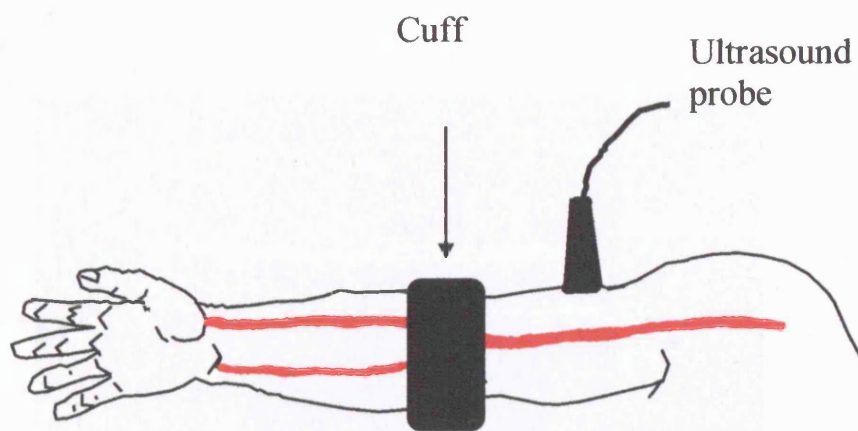
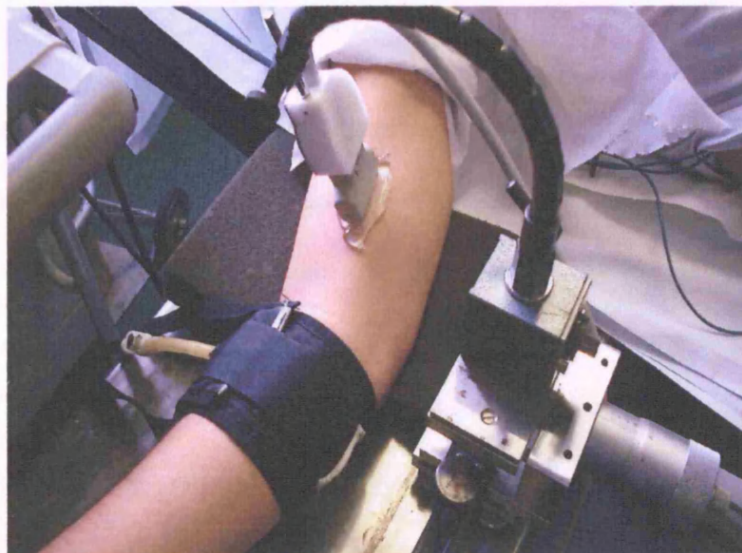


Figure 3.1A: Diagrammatic representation of technique



3.1 B: Photograph of the set up of the cuff and ultrasound probe

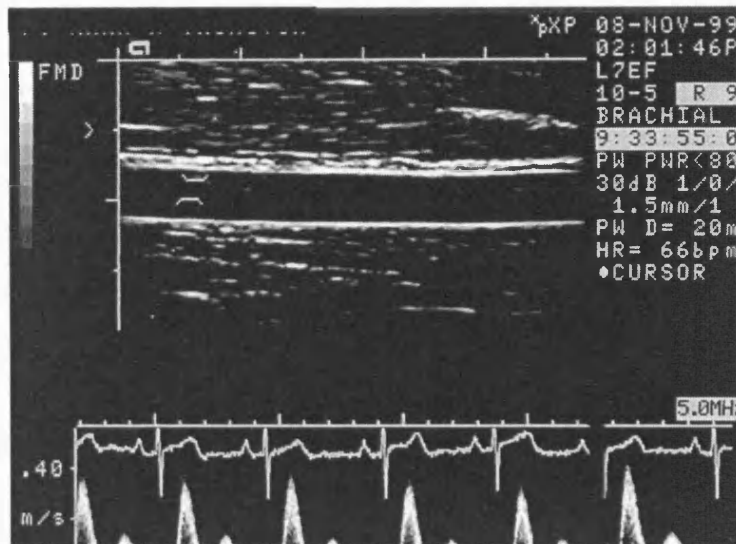


Figure 3.2: Static ultrasound image view of brachial artery, showing digital calipers within the arterial lumen. The calipers mark the point where blood flow is recorded. The Doppler signal measuring flow for each cardiac cycle is also shown in the lower part of the image.

End-diastolic images of the vessels were acquired every 3 seconds using data-acquisition software (Information Integrity, Boston, USA) and stored off-line for later analysis. Arterial diameter over a 1-2 cm segment was determined for each image using automatic edge detection software (Information Integrity, Boston USA). Analysis was performed by an experienced vascular technician blinded to the phase of the study. Using pulsed wave Doppler, blood flow was recorded continuously throughout the study and was expressed as the velocity time integral (VTI; area under the blood velocity/time curve for a complete cardiac cycle). Baseline recordings of arterial diameter were made for one minute before inflation of a blood pressure cuff placed distal to the site of arterial imaging. Recording continued for 5 minutes during cuff inflation to 300 mmHg and for 5 minutes after

deflation. Endothelium-independent dilatation of the brachial artery was assessed by measuring the dilator response to a 25µg dose of the NO donor, glyceryl trinitrate given sublingually. This elicited vascular dilatation of the same order of magnitude as that of the endothelium-dependent flow stimulus. Results are expressed as percentage maximum change in vessel diameter from baseline.

3.2.2 Blood flows

As the stimulus is flow, we needed to ensure that this was standardized so that the response of brachial artery dilatation could be compared between subjects and within subjects at different time points.

The Doppler signal from the observed vessel was the velocity-time profile for a single cardiac cycle and is displayed as a Doppler curve. The area under the curve was the velocity-time integral (VTI), and approximates to the average distance traveled by a pulse of blood during one cardiac cycle and was expressed as metres.

As the area under the curve (VTI) = velocity (metres/second) x seconds, so VTI = metres.

As volume of flow per minute is equal to the distance traveled multiplied by the cross-sectional area of artery and the heart rate, then VTI is proportional to volume flow since heart rate, the Doppler angle of incidence and arterial diameter remain essentially constant during the period of study.

Blood flow (VTI in m) produced by the cuff occlusion was recorded at baseline (5 seconds before cuff release), at the point of cuff release and at 5, 15, 30, 45, 60, 75 and 90 seconds post release of the cuff.

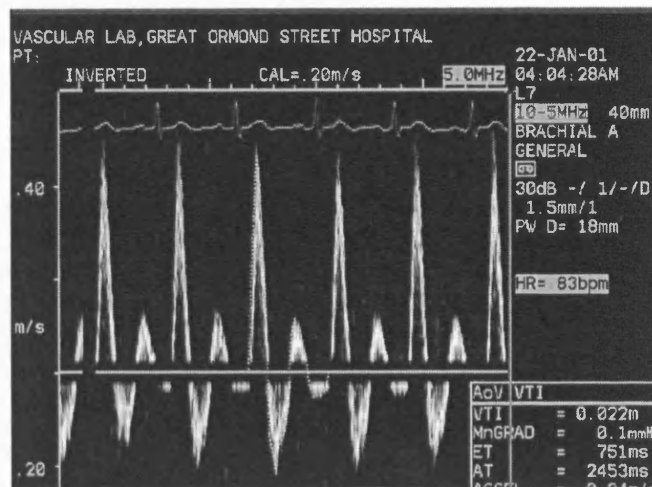


Figure 3.3A



Figure 3.3B

Figure 3.3 Examples of the Doppler curves from which flow (VTI=area under the curve) is calculated.

A: Pre release of the cuff VTI=0.22m

B: A time point post release of the cuff, VTI=0.403m

The peak of the increase in flow was recorded as maximum reactive hyperaemia and expressed as a percentage change from baseline.

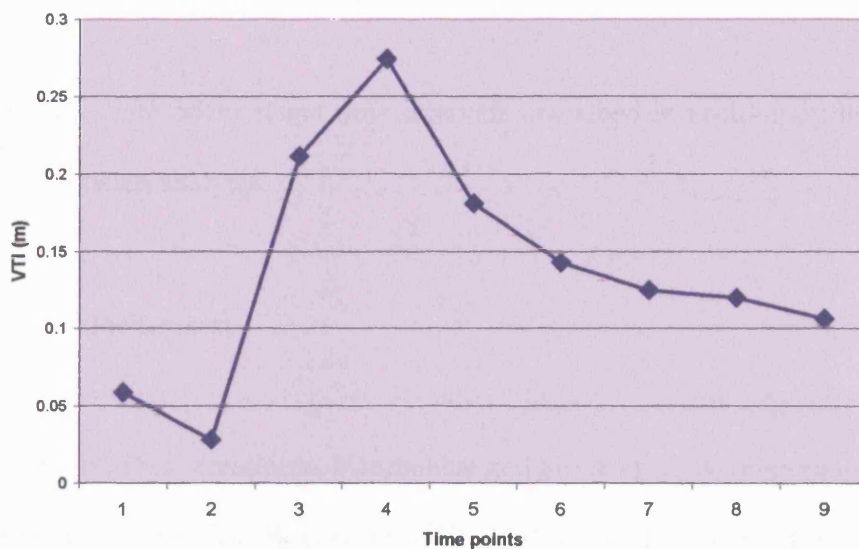


Figure 3.4: The change in blood flow recorded at the 9 time points.

3.2.3 Reliability and reproducibility

This technique has been reported to be both reliable and repeatable.

Sorensen et al investigated the possibility of error in the measurement of arterial diameter and noted using 'phantom' arteries that technicians were able to measure diameter correctly to within 0.04mm and could distinguish between pairs of phantom arteries with diameter differences 0.1 mm in 61% of cases (223).

Reproducibility in children aged 7 to 18yrs was investigated by Kari who recorded from analysis of variance a co-efficient of variation of 4.3% when measuring FMD on 3 different occasions by the same observer (3).

In adults Sorensen reported a co-efficient of variance for inter-observer repeatability of 1.8% for 3 different observers (223).

3.3 Biochemistry

Blood samples were taken at the time intervals described in each study by myself, spun and frozen for batch analysis.

3.3.1 General biochemistry

Full blood count, urea, creatinine, bicarbonate and electrolytes were measured (Vitros 750, Ortho-Clinical Diagnostics, Rochester, NY). Fasting lipid analyses were performed for total cholesterol (TC), high-density lipoprotein (HDL) and triglycerides (TGs) using colorimetric assays (Vitros 750, Orthoclinical). Both very low-density lipoprotein (VLDL) cholesterol and LDL were calculated. LDL subfractions were measured using high-resolution polyacrylamide gel electrophoresis (Quantimetrix, California) and reported as the ratio of less dense to more dense (LDL1+2:LDL3+4+5).

3.3.2 Measures of oxidative stress

Various indirect measures of oxidative stress have been developed and used extensively. Those used in the studies described in this thesis are, LDL lag times, total antioxidant activity, antibodies to oxidised LDL and lipid peroxides.

LDL is susceptible to oxidation and oxidised LDL is key in the development of atherosclerosis. LDL lag times are an in vitro measure of LDL resistance to oxidation, and were measured by firstly isolating LDL from heparinised plasma using density gradient ultracentrifugation and desalted by gel filtration. Oxidation was promoted using copper chloride. The production of conjugated diene (markers of lipid peroxidation) was monitored using spectrophotometry. Lag times were calculated as the time intercept between the line of maximum slope of the propagation phase and the absorbance at baseline equaled zero (see figure 3.5) (224).

Total antioxidant activity is an overall measure of the serums antioxidant capacity. The assay is based on the horseradish peroxidase-catalysed oxidation of a chemiluminescent substrate, luminol by hydrogen peroxide. The production of the light signal depends on continuous production of free radicals from luminol and oxygen. The signal can therefore be suppressed by free radical scavenging. The suppression will last until the test serums capacity is exhausted and the light signal will return. The result is calibrated against a standard solution (225).

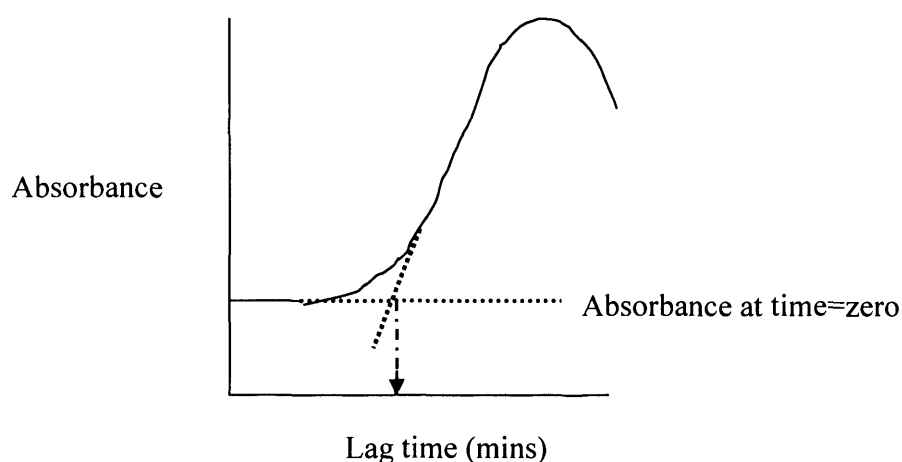


Figure 3.5: Method for determining lag time.

Antibodies to oxidised LDL

Antibodies against oxidatively modified LDL can be used as a measure of oxidation processes occurring in vivo. Elevated values have been found in subjects with atherosclerosis and correlations have been noted to the progression of vascular disease (226).

Antibodies to oxidised LDL were measured directly using an ELISA technique in a pre prepared kit (Olab, Biomedica Gruppe, Germany). Copper oxidised LDL was used as the antigen.

Total plasma lipid peroxides (reaction products of oxidative damage to lipids) by a lipid hydroperoxide assay kit (Cayman, San Diego).

L-Arginine and L-arginine analogues

Free L-arginine levels were measured by an automated amino analyser (Pharmacia, Milton Keynes, UK); plasma nitrite and nitrate (NO oxidation products) by chemi-luminescence; nitrated proteins (nitrotyrosines: NO and superoxide result in peroxynitrite, which combines with tyrosine residues and is present in atherosclerotic plaques) by competitive ELISA with nitrated human serum albumin as standard; and analogues of L-arginine, ADMA and SDMA by HPLC.

Folate levels and homocysteine

Serum and red cell folate levels were determined using radioimmunoassay (Abott IMx) with a normal range for serum folate of 2 to 20µg/L and red cell folate of 150 to 650µg/L. Plasma total (free and bound) homocysteine was measured using a competitive

fluorescence polarization immunoassay (normal range 4.4 to 13.7 μ mol/l for adults, Abbot IMx).

3.4 Statistics

The null hypothesis states that the intervention (L-arginine/folic acid) will have no extra effect on endothelial function over placebo. The study size was based on a power calculation at 80%, significance value of 0.05 to show a 2% improvement in FMD. Each subject served as his or her own control.

The data were tested for normality using Shapiro-Wilk and modified Kolmogorov-Smirnov test.

All descriptive data are expressed as group mean \pm standard deviation and significance is interpreted as a p value <0.05 .

The primary study endpoint was the change in FMD (post-treatment value minus pre-treatment value) on L-arginine/folic acid and on placebo. The change on L-arginine/folic acid was compared by paired Student t tests.

For the folic acid study a second analysis was performed, final FMD after folic acid and after placebo were compared using ANCOVA. All data were analysed for period and carry over effects.

Within subject variation in physical parameters was analysed using repeated measures linear model. Baseline values before active agent or placebo and other outcome variables were also compared using linear regression.

Chapter Four

The L-arginine study

4.1 Original hypothesis

Dietary supplementation with L-arginine (the substrate for NO synthetase) improves large vessel endothelium-dependent vasodilatation and NO bioavailability.

4.2 Introduction

Premature atherosclerosis is the most important cause of death in adults with chronic renal failure (CRF), and is believed to be due to a high incidence of the classical risk factors in this population (227). However, my group have previously demonstrated that conduit artery endothelial function is already abnormal in children with CRF even in the absence of these risk factors, suggesting a toxic effect of uraemia itself (3). Endothelial dysfunction is a key early event that precedes the formation of atherosclerotic plaques, and involves reduced bioavailability of nitric oxide (NO), an important anti-atherogenic agent (228). The semi-essential amino acid L-arginine is the only substrate for NO synthesis. Levels of L-arginine have been reported as being low in CRF but this is not a universal finding (229).

In CRF however, decreased clearance results in high levels of circulating analogues of L-arginine, asymmetrical dimethylarginine (ADMA) and its stereoisomer, symmetrical dimethylarginine, SDMA (85). ADMA has been characterised as endogenous inhibitor of NO synthase, the enzyme which catalyses the production of NO in vascular endothelial cells from L-arginine (86). Also, NO may be inactivated by increased oxidative stress and free radical production in CRF (see chapter 1.6). There are, therefore, several ways in which supplementation with L-arginine might improve the bioavailability of NO in CRF: it may correct substrate deficiency; overcome competitive antagonism; and increase

production to overcome rapid removal. Dietary L-arginine supplementation has already been shown to improve endothelial function in the brachial artery of young people with hypercholesterolaemia (152) and experimentally to inhibit atherogenesis in animal models (150,151).

My hypothesis was that arginine supplementation might improve NO bioavailability and endothelial function, and therefore lead to a simple preventative treatment strategy to decrease the risk of premature vascular disease. The technique of high-resolution ultrasound was used to measure endothelial function non-invasively by assessing the change in brachial artery diameter in response to endothelium dependent and independent stimuli, in a randomised, double blind placebo controlled cross-over trial of oral L-arginine supplementation in children with CRF.

4.3 Methods and subjects

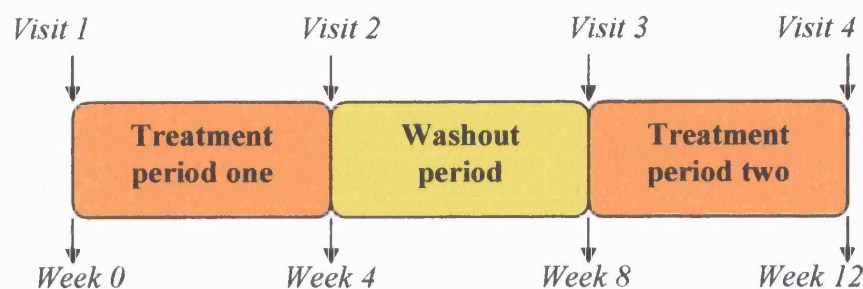
Subjects 4.2.1

Twenty-five children (11 girls and 14 boys, mean age 12 ± 3 years (range 7-17years)) with CRF ($\text{GFR} < 50 \text{ ml/min/1.73m}^2$) were studied. Twenty-four had congenital structural and one acquired (neonatal cortical necrosis) causes of CRF (see chapter 2). We did not preselect children on the basis of endothelial function in order that the study group should be representative of all children with moderate to severe CRF.

4.3.2 Study design

The trial was randomised, placebo controlled, double blinded and cross over in design with two four-week treatment periods separated by a four-week washout period. Blood samples were taken following a 6 hour fast. L-arginine was given at $5\text{g}/\text{m}^2$ surface area to a maximum of 7 gms three times daily. This dose was selected to provide a two fold increase in plasma L-arginine levels and was based on previous studies performed by our group (8). After 3 children with low GFR complained of nausea and were found to have an elevated urea on completion of the first treatment phase the dose of the treatment syrup was subsequently adjusted in those with a $\text{GFR} < 35\text{mls}/\text{min}/1.73\text{m}^2$ to $2.5\text{g}/\text{m}^2$ three times daily (without unblinding the study). The active drug preparation and the placebo were prepared as syrup by South Devon Healthcare, Paignton, Devon TQ4 7TW. Dietary intake of nitrates and nitrites were not restricted as it was thought not to be practical in this age group and we anticipated poor compliance. As each child acted as their own control, it was not felt to be a confounding variable. However, this places limitations on the interpretation of the nitrate/nitrite data.

Children were evaluated at the start and end of each treatment period. At each visit, supine blood pressure was recorded, blood taken (after a 6 hour fast) and vascular function was assessed.



4.3.3 Assessment of vascular function

Endothelial function was determined by recording the dilator response of the brachial artery to increase blood flow generated during reactive hyperaemia of the downstream forearm, flow mediated dilatation (FMD). The technique is as described in chapter 3.2. Analysis was performed by an experienced vascular technician blinded to the phase of the study.

4.3.5 Laboratory assays

Urea, creatinine, bicarbonate and electrolytes were measured. Fasting lipid analyses were performed for total cholesterol (TC), high-density lipoprotein (HDL) and triglycerides (TGs). Both very low-density lipoprotein (VLDL) cholesterol and LDL were calculated. LDL subfractions were measured using high-resolution polyacrylamide gel electrophoresis. LDL lag times, lipid peroxides and antioxidant activity were measured as described in chapter 3.3.

Free L-arginine levels were measured by an automated amino analyser; plasma nitrite and nitrate (NO oxidation products) by chemi-luminescence; nitrated proteins (nitrotyrosines: NO and superoxide result in peroxynitrite, which combines with tyrosine residues and is present in atherosclerotic plaques) and analogues of L-arginine, ADMA and SDMA by HPLC.

Power calculations were as described in chapter 3. The primary study endpoint was the change in FMD (post-treatment value minus pre-treatment value) on L-arginine and on placebo. The change on L-arginine and placebo was compared by paired Student t tests.

4.4 Results

4.4.1 Baseline characteristics

The clinical and biochemical characteristics of the study group are shown in table 4.1. Twenty-one children completed the study. One child received a renal transplant and three were unable to tolerate the L-arginine due to unpleasant taste in two and nausea in one.

	Mean value \pm SD
Age (years)	11.5 \pm 3
Sex ♂:♀	11:10
Ht (cms)	142.4 \pm 20.9
Weight (Kg)	40.3 \pm 15.8
Systolic/Diastolic BP (mmHg)	113 \pm 14/65 \pm 8
GFR (nr 80-120 mls/min/1.73m ²)	27.4 \pm 13.2
Serum creatinine (nr 30-102 μ mol/l)	258 \pm 210
L-arginine (nr 40-120 μ mol/l)	79 \pm 20.2
Serum total cholesterol (nr 3.1-5.4 mmol/l)	4.95 \pm 1.16
Serum triglycerides (nr 0.4-1.4mmol/l)	1.5 \pm 0.54

Table 4.1: Physical and biochemical characteristics at entry of the 21 children who completed the L-arginine study. The normal range (nr) is given where appropriate.

4.4.2 Effect of L-arginine on biochemistry (Table 4.2)

Group mean baseline levels of L-arginine were normal and no single value was below the lower limit. The dose of L-arginine used produced a significant rise in serum levels during the treatment phase when dosing regimen were pooled ($p < 0.001$) compared with placebo ($p = 0.82$). There was no significant difference in the post treatment levels of L-arginine on either dosing regimen. In the treatment phase versus placebo phase a rise in urea (change in

urea 3.92 ± 4.94 versus 0.85 ± 2.68 mmols/l, $p=0.015$), a fall in bicarbonate (change in bicarbonate -3.05 ± 3.84 versus 0.43 ± 2.42 mmol/l $p=0.006$), but no significant change in creatinine ($p=0.87$) were noted. All these values returned to baseline during the washout period for those who received L-arginine first.

	Baseline	Post L-arginine	Baseline	Post placebo	Change on L-arginine	Change on placebo	
Urea (nr 2.2-5.7 mmol/l)	12.2 ± 7.8	16.1 ± 11.1	11.6 ± 6.0	12.5 ± 7.4	3.9 ± 4.9	0.9 ± 2.7	0.015
Bicarbonate (nr 20-26 mmol/l)	22 ± 3	19 ± 3	22 ± 2	23 ± 2	-3. ± 4	0.4 ± 2.4	0.006
Creatinine (nr 0-120 μ mol/l)	256 ± 215	270 ± 233	254 ± 210	270 ± 234	14 ± 42	15 ± 31	ns

Table 4.2: Effect of L-arginine supplementation on renal chemistry. Data are expressed as mean \pm SD. P values for change on L-arginine v change on placebo.

4.4.3 Lipid analysis (Table 4.3)

Baseline total cholesterol were normal but triglycerides were elevated. There was a significant fall in TGs on treatment versus placebo (-0.06 ± 0.54 versus 0.19 ± 0.57 mmol/l, $p=0.005$) otherwise lipid profiles remained unchanged.

4.4.4 Oxidative stress (Table 4.3)

A highly significant fall in total antioxidant activity of the serum was noted during the treatment period versus the placebo phase (-41 ± 30 versus 16 ± 54 microM trolox Eq, $p < 0.001$). LDL lag times (a measure of the susceptibility of LDL to oxidation) and antibodies to oxidised LDL were above the normal range for adults at baseline and there was no significant change with treatment. There was no effect of L-arginine on the ratio of low density to high density LDL or lipid peroxides.

	Baseline	Post L- arginine	Baseline	Post placebo	Change on L-arginine	Change on placebo	
Antibodies to oxidised LDL (n<250 mU/ml)	631 ±501	579 ±468	609 ±406	562 ±478	-23 ±72	-47 ±91	ns
LDL Lag times (n >60 mins)	87±19	83±21	89±17	89±20	0.2±18	0.5±23	ns
Lipid peroxides (µMol)	17.3 ±21.2	16.6 ±24	13.7 ±23.2	22.9 ±34.5	-0.7 ±13.9	9.2 ±30.5	ns
Antioxidant activity (n>440µM trolox Eq)	250±97	202±84	226±90	242±98	-41±30	16±54	<0.001
Lipoprotein ratios	5.46 ±3.43	6.57 ±4.58	7.37 ±6.0	6.46 ±5.7	1.62 ±5.45	-0.9 ±7.53	ns
TC (n 3.1-5.4 mmol/l)	4.8 ±1.3	4.5±1.1	4.9±1.0	4.8±0.9	-0.1±0.7	-0.2±0.5	ns
TG (n 0.4 –1.4 mmol/l)	1.53 ±-0.53	1.45 ±0.7	1.51 ±0.55	1.96 ±0.74	-0.06 ±0.54	0.19 ±0.57	0.005
HDL (n>0.91mmol/l)	1.3±0.4	1.2±0.4	1.1±0.4	1.2±0.3	-0.01±0.2	0.04±0.24	ns
LDL (n<3.3mmol/l)	2.57 ±1.2	2.45 ±1.0	3.03 ±0.85	2.72 ±0.9	0.36 ±1.75	-0.27 ±0.62	ns

Table 4.3: Effect of L-arginine and placebo on lipid chemistry. Normal values (n) given where available. Data are expressed as mean±SD. P-value is given for change on L-arginine versus change on placebo.

4.4.5 Nitrate chemistry (Table 4. 4)

Plasma nitrate was elevated but plasma nitrite was normal at baseline. Plasma nitrated protein (nitrotyrosine) levels were increased significantly at entry to the study when compared with data available on normal children (0.126 ± 0.07 v 0.02 ± 0.007 Nitro-BSA equivs microgram/ml/mg protein, $p < 0.001$). No significant changes in plasma nitrate or nitrite were observed with L-arginine treatment. Protein nitrotyrosine concentrations increased with L-arginine treatment but not significantly. There was no relationship between nitrotyrosine concentrations and those of nitrate and urea. Both L-arginine analogues ADMA and SDMA were elevated at baseline but there was no significant change on treatment or placebo.

A negative correlation between SDMA and GFR was the only relationship noted with $r^2 = 0.6$ and $p < 0.05$. Otherwise no significant relationships between NO metabolites and GFR were seen.

	Baseline	Post L- arginine	Baseline	Post placebo	Change on L- arginine	Change on placebo	
Nitrates (n 32.1± 4.3µMol)	68.6±63.1	67.2±37.4	70.0±55.6	62.9±39.3	-1.13±59.4	-7.12±67.1	ns
Nitrites (n 1.4±0.2µMol)	1.66±0.77	1.09±0.54	1.72±0.85	1.29±2.2	-0.57±0.8	-0.43±2.47	ns
L-arginine* (n 80- 120µMol/l)	82.9±20	179±110	72.6±19.7	70.7±21.2	104±110	4±14.5	<0.001
ADMA (n 0.7± 0.1µMol/l)	1.31±0.55	1.19±0.60	0.98±0.48	0.84±0.41	-0.02±0.91	0.024 ±0.13	ns
SDMA (n 0.3± 0.1µMol/l)	2.16±1.41	1.88±1.54	1.91±1.37	1.90±1.87	-0.58±1.66	-0.45±0.93	ns
Nitrotyrosines (n 0.02±0.007 NT – BSA equivs, Microgram/ml/g)	0.12±0.7	0.16±0.11	0.11±0.07	0.12±0.06	0.04±0.1	0.004 ±0.05	ns

Table 4.4: Effect of L-arginine on NO chemistry. Results are expressed as mean±SD.

Normal values (n) for nitrates, nitrites, ADMA and SDMA are quoted from published data

(2). * Result of pooled doses. P-value is given for change on L-arginine versus change on placebo.

4.4.6 Effect of oral L-arginine on vasomotor function (Table 4.5)

There was no relationship between baseline FMD and any clinical and biochemical parameters measured. There was no effect of L-arginine on baseline arterial diameters, baseline blood flow or reactive hyperaemia ($p=ns$) on within subject analysis (hence no replication error), using the linear model repeated measures analysis.

	Baseline	Post L-arginine	Baseline	Post placebo	Change on L-arginine	Change on placebo	
Brachial artery diameter (mm)	3.28 ±0.62	3.28 ±0.69	3.28 ±0.63	3.24 ±0.69	0.007 ±0.15	-0.03 ±0.18	ns
Baseline flow (VTI, m)	0.06 ±0.03	0.07 ±0.05	0.08 ±0.04	0.07 ±0.36	0.004 ±0.03	0.001 ±0.04	ns
Maximum reactive hyperaemia (%)	529±208	471±243	489±333	480±249	-55.2±228	-8.76±364	ns
FMD (%)	7.94 ±2.26	7.80 ±2.95	8.2 ±2.81	8.24 ±3.12	0.06 ±2.4	-0.14 ±3.23	ns
Systolic blood pressure (mmHg)	113±14	107±11	112±15	111±16	-4.2±6.5	1±11.8	ns
Diastolic blood pressure (mmHg)	64±10	61±10	63±10	65±9	-3.3±10.4	3.3±15.8	ns

Table 4.5: Vascular responses to L-arginine and placebo. Data are expressed as mean ±SD.

Flow mediated dilatation (Endothelial dependent dilatation) (Figure 4.1)

No significant difference in the change in flow mediated dilatation during the treatment phase versus the placebo phase was noted (p=0.84).

Response to GTN (Endothelial independent dilatation) (Figure 4.1)

No significant difference in the change in response to GTN during the treatment phase versus the placebo phase was noted ($p=0.2$).

There was no significant change in resting heart rate, supine blood pressure during treatment or placebo phase suggesting that there was no haemodynamic effect of the L-arginine.

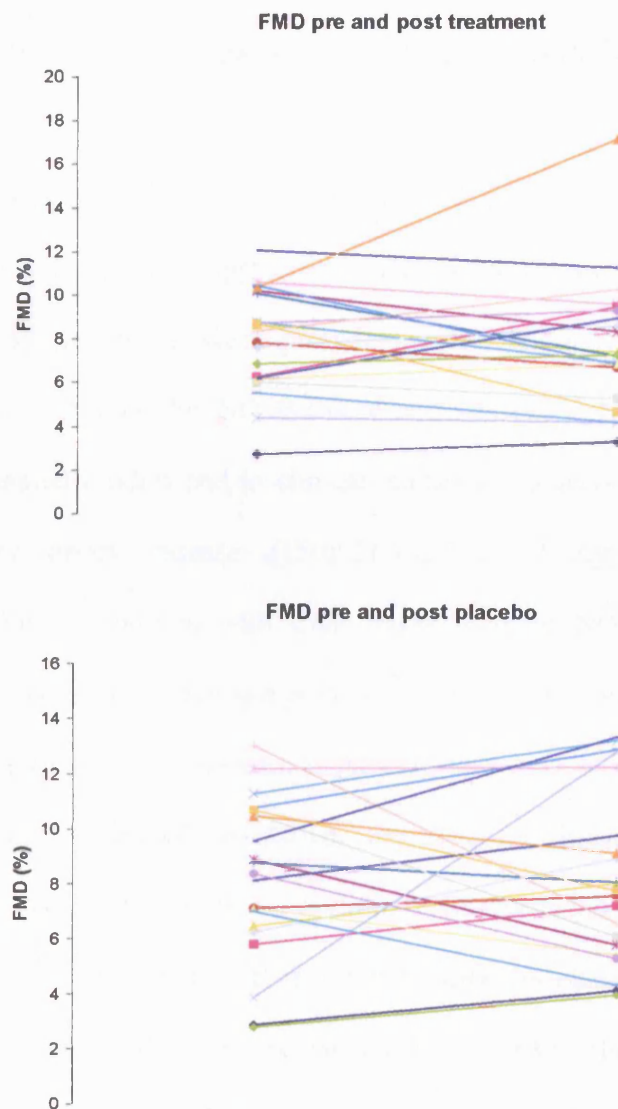


Figure 4.1: FMD pre and post treatment with L-arginine and placebo. $P=ns$ for group means.

4.5 Discussion

This study has shown that oral supplementation with L-arginine did not improve brachial artery FMD, a measure of endothelial derived NO bioavailability, in children with CRF, despite achieving plasma levels of twice normal. Indeed, it would not have been possible to increase the dose any further as the children developed a significant increase in plasma urea, a fall in venous bicarbonate and a fall in total antioxidant activity.

The biological activity of NO is impaired in CRF (114) which leads to reduced vascular relaxation, increased platelet aggregation, increased leucocyte adhesion and smooth muscle proliferation all processes which precede the formation of atherosclerotic plaques. L-arginine is the substrate for NO synthase and has been shown to increase endothelial function in animal models and in clinical studies of subjects with hypercholesterolaemia and coronary artery disease (150,151,152,155). I hypothesised that L-arginine supplementation in children with CRF might increase NO bioavailability by 3 main mechanisms: firstly by correcting a possible substrate deficiency; secondly by reducing the effect of endogenous inhibitors such as ADMA, known to be elevated in CRF; and thirdly by overcoming increased oxidative inactivation. Substrate deficiency was not demonstrated, as baseline serum L-arginine levels were normal. However, intracellular levels may not correlate with serum levels in CRF, as elevated levels of oxidised LDL (which are present in CRF) are associated with defective cellular transport for L-arginine (230).

L-arginine supplementation might overcome the effect of elevated methylated analogues, ADMA and SDMA in CRF. ADMA is an inhibitor of eNOS (86) and although SDMA is not a direct inhibitor it competes with the cationic amino acid transporter in the endothelial

cell membrane (231) and therefore could heighten any intracellular arginine deficiency. In these children, we found both SDMA and ADMA were elevated at baseline when compared with previously published data in children (3). Levels were, however, not as high as those previously reported in adults with CRF (85, 86). The modest elevation of ADMA in this study group may have contributed to the lack of vascular benefit seen with L-arginine supplementation. There was, however, no correlation between the response of FMD and levels of ADMA or those of L-arginine.

Markers of oxidative stress (antibodies to oxidised LDL, antioxidant activity) were elevated in this study. Increased oxidative stress might be responsible for reducing NO bioavailability by conversion of NO to nitrate, nitrite, and peroxynitrite (a potent cytotoxic agent) by superoxide. L-arginine supplementation could overcome this as in vitro studies have shown that L-arginine supplementation prevents preferential superoxide production in endothelial cells exposed to near physiological levels of oxidised-LDL (84). In these CRF children, there was no biochemical evidence of increased NO production (nitrate and nitrite levels remained unchanged) despite a doubling of the L-arginine levels. In view of the unrestricted diet this observation should be interpreted with caution. De Nicola et al achieved similar L-arginine levels in adult CRF patients with oral supplementation and were also unable to demonstrate enhanced NO production by measuring urinary cyclicGMP (intracellular mediator of NO). It is possible that important co-factors for eNOS such as tetrahydrobiopterin may have been deficient in the patients in this study (223). Under these conditions, NOS preferentially reduces molecular oxygen to superoxide even in the presence of adequate substrate concentrations. L-arginine at the dose used had adverse metabolic effects including elevated urea and extra cellular acidosis. These could have affected pH dependent signaling pathways and, therefore, eNOS activity. There was, however, no correlation between these fully reversible effects and endothelial function.

The reduction in total antioxidant activity of the serum suggests that increased oxidant stress was generated during the treatment phase.

L-arginine supplementation in this group of children with CRF did not improve endothelial function. Oral supplementation at a dose producing a two-fold rise in serum levels resulted in adverse metabolic sequelae in several children, and may have contributed to the negative outcome. Investigation into other mechanisms of impaired vascular biology in CRF is required.

Chapter Five

Folic acid study

5.1 Original hypothesis

Dietary supplementation with folic acid reduces homocysteine levels and improves large vessel endothelium-dependent dilatation.

5.2 Introduction

In CRF, homocysteine is also an independent risk factor (99) for cardiovascular disease and in dialysis patients, hyperhomocysteinaemia is more prevalent than traditional cardiovascular risk factors (100). Homocysteine may, therefore, be contributing to aggressive “accelerated atherosclerosis” in CRF. In vitro and in vivo studies suggest that homocysteine causes endothelial dysfunction either directly or via intermediate reactions increasing oxidised LDL levels (101,102).

Folic acid has been shown to lower homocysteine levels in several populations and can improve endothelial function (163-166). In CRF there appears to be relative resistance to folic acid, but supplementation in adults with doses of 5-15mg per day can decrease homocysteine levels by as much as 40-50% (165). The effect on endothelial function has however been less impressive (175-177).

In this study high-resolution ultrasound was used to study the effect of folic acid supplementation on homocysteine and vascular function in children with moderate to severe CRF. Children were selected specifically both to reduce the influence of confounding factors and thus provide a clinical model of uraemic influences on the arterial wall, and to determine whether early intervention might have greater vascular benefits than those seen in adults.

5.3 Subjects and methods

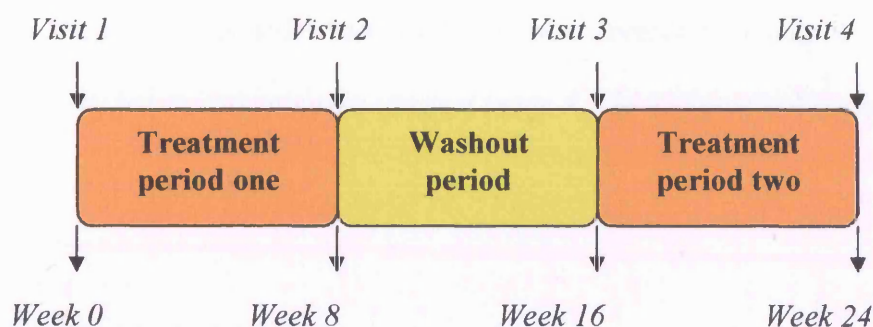
5.3.1 Subjects

Twenty-five children (11 girls and 14 boys, mean age 12 ± 3 years (range 7-17)) with CRF ($\text{GFR} < 50 \text{ ml/min/1.73m}^2$) were recruited from the outpatient department at Great Ormond St Hospital. Twenty-four had congenital structural and one acquired (cortical necrosis) causes of CRF (see Chapter 2.2). No child received folic acid supplementation or vitamins (apart from activated vitamin D) prior to the study.

5.3.2 Study design

I performed a randomised, placebo-controlled, double-blinded, crossover trial with two eight-week treatment periods separated by an eight-week washout period. Folic acid was given at a dose of 5 mg/m^2 surface area (Special products Ltd Addlestone, Surrey KT15 1TU, who also prepared the placebo).

Children were evaluated at the start and end of each treatment period. At each visit, supine blood pressure was recorded, blood taken (after a 6 hour fast) and vascular function was assessed.



5.3.3 Assessment of vascular function

Endothelial function was determined by recording the dilator response of the brachial artery to increase blood flow generated during reactive hyperaemia of the downstream forearm, flow mediated dilatation (FMD). The technique is as described earlier chapter 3 section 3.2. Analysis was performed by an experienced vascular technician blinded to the phase of the study.

5.3.4 Laboratory assays

Full blood count, urea, creatinine, bicarbonate and electrolytes were measured. Fasting lipid analyses were performed for total cholesterol (TC), high-density lipoprotein (HDL) and triglycerides (TGs). Both very low-density lipoprotein (VLDL) cholesterol and LDL were calculated. LDL subfractions were measured using high-resolution polyacrylamide gel electrophoresis. LDL lag times, lipid peroxides and antioxidant activity were measured as described in chapter 3.

Serum and red cell folate levels were determined using radioimmunoassay (Abott IMx) with a normal range for serum folate of 2 to 20µg/l and red cell folate of 150 to 650µg/l. Plasma total (free and bound) homocysteine was measured using a competitive fluorescence polarization immunoassay (normal range 4.4 to 13.7µmol/l for adults) (Abbot IMx).

Power calculations were as described in chapter 3. The primary study endpoint was the change in FMD (post-treatment value minus pre-treatment value) on folic acid and on placebo. The change on folic acid and placebo was compared by paired Student t tests.

In addition final FMD after folic acid and after placebo were compared using ANCOVA.

All data were analysed for period and carry over effects. All descriptive data are expressed as group mean \pm standard deviation and significance is interpreted as a p value <0.05 .

5.4 Results

5.4.1 Baseline characteristics

The clinical and biochemical characteristics of the study group are shown in table 5.1.

Twenty-three children completed the study. One child was transferred to peritoneal dialysis and one received a renal transplant.

	Mean value \pm SD
Age (years)	11.5 \pm 3
Sex ♂:♀	13:10
Ht (cms)	144.3 \pm 17.9
Weight (Kg)	40.9 \pm 14.7
Systolic/Diastolic BP (mmHg)	110 \pm 10/67 \pm 9
GFR (nr 80-120 mls/min/1.73m ²)	28.3 \pm 12.7
Serum creatinine (nr 30-102 μ mol/l)	229 \pm 193
Total homocysteine (nr 4.4-13.7 μ mol/l)	9.85 \pm 3.57
Serum total cholesterol (nr 3.1-5.4 mmol/l)	4.74 \pm 1.05
Serum triglycerides (nr 0.4-1.4mmol/l)	1.66 \pm 0.65
Haemoglobin (nr 13-16g/dl)	12.8 \pm 1.49

Table 5.1: Physical and biochemical characteristics at entry of the 23 children who completed the folic acid study. Normal range (nr) given where appropriate

5.4.2 Effect of folic acid (Table 5.2)

There was no effect of folic acid on haemoglobin or renal function. At entry to the study, serum folate (13.7 \pm 3.58 μ g/l) and red cell folate levels (334 \pm 202 μ g/l) were normal. Folic acid produced a significant increase in both serum folate (11.7 \pm 4.25 to 635 \pm 519 μ g/l p=0.001) and red cell folate (364 \pm 195 to 2891 \pm 2623 μ g/l p<0.001) levels during the treatment period.

During placebo, there was no change in serum or red cell folate levels when the placebo phase preceded the folic acid phase (13.6 \pm 4.6 to 10.68 \pm 5.76 and 348 \pm 244 to 351 \pm 127 μ g/l

p=ns). However, in the children who received placebo after folic acid, the serum folate changed from 20 ± 9.9 to $14.01 \pm 6.08 \mu\text{g/l}$ (p=ns) and the red cell folate from 820 ± 517 to $470 \pm 185 \mu\text{g/l}$ (p=0.02) during the placebo phase. These post placebo levels were higher at the end of the study than at entry suggesting a carry over effect for red cell folate.

5.4.3 Homocysteine levels (Table 5.2)

Homocysteine levels at entry to the study were greater ($9.85 \pm 3.57 \mu\text{mol/l}$) than published data on normal children. There was a significant fall in total homocysteine levels after folic acid (10.28 ± 4.16 to $8.62 \pm 2.32 \mu\text{mol/l}$ p=0.03), but not in the placebo phase (9.02 ± 2.19 to $9.84 \pm 2.7 \mu\text{mol/l}$ p=0.3).

5.4.4 Lipid analysis (Table 5.2)

Baseline total cholesterol levels were within the normal range ($4.74 \pm 1.05 \text{mmol/l}$) and there was no significant change with treatment or placebo. Triglycerides were elevated above the normal range ($1.66 \pm 0.65 \text{mmol/l}$) and were unchanged after folic acid or placebo. HDL and LDL cholesterol were within the normal range at baseline ($1.36 \pm 0.36 \text{mmol/l}$ (normal range, 0.93-1.94) and $2.7 \pm 0.8 \text{mmol/l}$ (normal range, 1.63-3.63) respectively and did not change significantly with either treatment or placebo.

5.4.5 Oxidant stress (Table 5.2)

Baseline values for LDL lag times were within the normal range. There was a significant increase in LDL lag times after folic acid (58.4 ± 18.7 to $68.1 \pm 25.9 \text{mins}$ p=0.01) compared

to placebo (62.8 ± 17.4 to 63.2 ± 13.3 mins $p=0.92$) suggesting that folic acid supplementation reduced susceptibility of LDL to oxidation. Ratios of low to high density LDL (25 ± 37 to 24 ± 34 $p=ns$) remained unchanged during treatment and placebo phases (22 ± 32 to 30 ± 36 $p=ns$) as did total serum antioxidant activity (204 ± 80 to 208 ± 74 on treatment v 188 ± 65 to 216 ± 74 μ trolox Eq on placebo $p=ns$).

	Baseline	Post- placebo		Baseline	Post FA	
SFA (nr 3-20 μ mol/l)	17.0 ± 8.9	12.4 ± 6.0	ns	13.1 ± 8.8	635 ± 519	0.001
RCF (nr 150-650 μ mol/l)	596 ± 468	405 ± 168	0.02	364 ± 195	2891 ± 2623	0.0004
Total homocysteine (nr 4.4-13.7 μ mol/l)	9.02 ± 2.19	9.84 ± 2.74	ns	10.28 ± 4.16	8.62 ± 2.32	0.03
Total Antioxidant activity (n 440 μ trolox Eq)	188 ± 66	216 ± 74	ns	203 ± 80	207 ± 74	ns
LDL lag times (n 60 mins)	62.8 ± 17	63.2 ± 13	ns	58.4 ± 18	68.4 ± 25	0.001

Table 5.2: Biochemical responses to folic acid (FA) and placebo. Results are given as mean \pm SD. Normal ranges (nr) in brackets.

5.4.6 Effect of folic acid on vasomotor function (Figure 5.1)

There was no significant change in baseline arterial diameter, baseline arterial flow or peak reactive hyperaemia after folic acid or placebo (table 5.3).

Endothelial dependent dilatation (FMD)

A significant improvement in FMD, expressed as percentage and absolute change in vessel diameter (7.21 ± 2.81 to $8.47 \pm 3.01\%$ $p=0.036$ and 0.217 ± 0.106 to 0.252 ± 0.081 cms $p=0.47$) was seen after folic acid, which was not seen after placebo (8.20 ± 3.41 to $8.80 \pm 4.01\%$ $p=0.44$ and 0.244 ± 0.102 to 0.276 ± 0.104 cms $p=0.14$). There was, however, no statistically significant difference in post-treatment FMD after placebo or folic acid ($p=ns$). Mean time of maximum dilatation after cuff release was not significantly different before or after treatment phases (pre-placebo 54 ± 16 seconds, pre-folic acid 59 ± 13 seconds, post-placebo 65 ± 19 seconds, post-folic acid 66 ± 17 seconds). No carry over or period effect on FMD was detected ($p=0.2$ and 0.17 respectively).

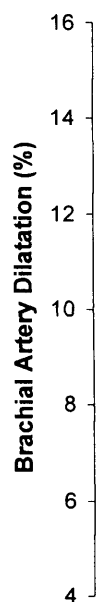
Endothelial independent dilatation (GTN)

There was no significant change in response to GTN on either folic acid (12.59 ± 6.5 to $11.58 \pm 5.39\%$ $p=0.28$ and 0.374 ± 0.136 to 0.35 ± 0.129 cms $p=0.4$) or placebo (12.93 ± 5.71 to $13.75 \pm 6.46\%$ $p=0.32$ and 0.390 ± 0.119 to 0.404 ± 0.170 cms $p=0.5$).

There was no significant change in resting heart rate or supine blood pressure after folic acid or placebo.

	Baseline	Post-placebo		Baseline	Post FA	
FMD (%)	8.2±3.42	8.80±4.01	ns	7.21±2.8	8.49±3.02	0.036
FMD (cms)	0.244	0.276	ns	0.217	0.252	0.047
	±0.102	±0.104		±0.106	±0.081	
GTN (%)	12.93±5.71	13.75±6.46	ns	12.59±6.53	11.58±5.39	ns
GTN (cms)	0.390	0.404	ns	0.374	0.350	ns
	±0.119	±0.170		±0.136	±0.129	
Arterial diameter (mm)	3.11±0.57	3.18±0.59	ns	3.13±0.56	3.13±0.58	ns
Resting blood flow (VTI, m)	0.058±0.03	0.072±0.03	ns	0.065±0.04	0.074±0.04	ns
Peak reactive hyperaemia (%)	680±540	464±233	ns	488±221	494±232	ns

Table 5.3: Vascular responses to folic acid (FA) and placebo. Results are given as mean±SD.



Change in mean (\pm SEM) brachial artery dilatation (■= GTN p =ns, ▲=FMD p =0.036) on folic acid.



Pretreatment

Post treatment

Change in mean (\pm SEM) brachial artery dilatation (■= GTN p =ns, ▲=FMD p =ns) on placebo.

Figure 5.1: Changes in mean brachial artery dilatation in response to flow (FMD and GTN) on folic acid and placebo

5.5 Discussion

This study shows that in children with CRF, supplementation with high dose folic acid for 8 weeks results in reduction in homocysteine levels, decrease in LDL susceptibility to oxidation and improvement in endothelial function. These encouraging findings contrast with the disappointing effects of folic acid supplementation on vascular function in adults with renal disease.

Homocysteine levels are consistently elevated in adults with CRF and this has been suggested to play a role in the pathogenesis of atherosclerosis especially in view of its strong association with death from vascular disease in the non-uraemic population (95, 96, 97, 98). A high prevalence of other risk factors exists in CRF but an independent association has been found between elevated total homocysteine levels and the risk of myocardial infarction (9). The data on homocysteine in children is limited. Lilien et al (93) reported elevated total homocysteine levels (12.6 ± 5.2 v $8.2 \pm 3.3 \mu\text{mol/l}$ $p=0.004$) in CRF children compared to controls. Total homocysteine levels at entry to our study ($9.85 \pm 3.57 \mu\text{mol/l}$) were also elevated in comparison to these controls.

Homocysteine levels can be lowered using folic acid. This increases tissue methylation of homocysteine to form methionine in both uraemic and non-uraemic individuals, even in the presence of normal folate levels. Studies in adults with hyperhomocysteinaemia and hypercholesterolaemia have shown improvement in endothelial function as a consequence of lowering total homocysteine using folic acid (166,167,168). Similar studies in CRF have been disappointing. In both CRF and those on dialysis, no improvement in endothelial

function has been demonstrated despite significant reductions in homocysteine. Thambyrajah et al (175) published a prospective double-blind trial in which 100 adults with a mean GFR of 30mls/min and a baseline total homocysteine of 20.1 μ mol/l were randomised to either folic acid or placebo. They achieved mean serum folate levels of 39 μ g/l and red cell folate levels of 739 μ mol/l with 5mg of folic acid for 12 weeks. These values were lower than those achieved in this study. No improvement in endothelial function (using FMD) was seen despite a significant reduction in total homocysteine. Van Guldener et al (175) treated 30 adults on peritoneal dialysis for 12 weeks with 5 mg folic acid alone or together with 4g betaine (an additional co-factor) followed by 1 or 5 mg folic acid for 40 weeks. Total homocysteine levels were grossly elevated (42.6 μ mol/l) at the beginning of the study and normalised in 40% of patients without any improvement in FMD. In a further attempt to demonstrate long-term clinical benefit from folic acid administration, no improvement in endothelial function was seen after 52 weeks in adult haemodialysis patients, despite a significant reduction in homocysteine levels (177). Similarly in another population of adults on haemodialysis, carotid artery distensibility and compliance did not change after folic acid supplementation (232). The explanation for these largely negative studies may be due to the particularly aggressive complex nature of the vascular disease, the inability to normalise homocysteine levels in CRF (165,233), abnormal folate metabolism (234) or inadequate folate supplementation.

The dose of folic acid in this study produced serum and red cell folate levels higher than in most published clinical intervention studies on CRF patients in the literature, in which endothelial function was the primary endpoint. Variations between 1mg and 60mg daily have been used in the renal adult literature with no extra benefit on homocysteine levels conferred by the higher doses. Duration of treatment in adult studies varied from 4 weeks

to 52 weeks with the maximum effect on homocysteine seen in the first two weeks and no further lowering despite increasing doses of folic acid (233)

At the end of the folic acid treatment period homocysteine levels had fallen significantly. There was an 8-week washout period between the treatment phases. Analysis of serum and red cell folic acid levels showed that the subjects who received placebo after the active phase had a fall in red cell folate levels. This implies that there was a “carry over” from the active phase and that ideally the washout period could have been longer. There was, however, no carry over effect on homocysteine levels.

There was a significant improvement in FMD during the folic acid treatment phase without change in response to GTN suggesting a beneficial effect of folic acid on endothelial function after eight weeks of treatment. It should, however, be noted that the final FMD after placebo and active phases were not significantly different. These findings must therefore be interpreted with caution and a longer-term trial may be warranted.

The mechanism by which homocysteine exerts its toxic affect on the endothelium is thought principally to be due to the generation of free radical species (101). In experimental hyperhomocysteinaemia induced by methionine infusion in volunteers, vitamin C improved endothelial function (102). In this study, there was a significant fall in total homocysteine levels with folic acid in parallel with an increase in LDL resistance to oxidation, through measurement of lag times. Total antioxidant activity was also measured but no significant change was noted. Thus increasing the resistance of LDL oxidation

might play an important role in the improvement in endothelial function, as oxidised-LDL is a potent vascular toxin. Alternatively folate may improve endothelial function via endogenous regeneration of tetrahydrobiopterin an essential co-factor in NO production or through a direct antioxidant effect as shown in vitro (235, 174).

Folic acid is safe, lowers homocysteine, reduces LDL susceptibility to oxidation and may improve endothelial biology relevant to the development of atherosclerosis. Long-term benefits require further study.

Chapter Six

Haemodialysis study

6.1 Original Hypothesis

Haemodialysis improves large vessel endothelium-dependent dilatation by lowering homocysteine levels, removing inhibitors of NO synthesis, reducing oxidative stress and/or removing free radicals.

6.2 Introduction

Although the aim of management for children in end-stage renal disease (ESRD) is transplantation, for many periods of dialysis are necessary. It has been postulated that the process of haemodialysis may, in itself, be atherogenic (236, 237). One mechanism for this may be through reduced bioavailability of NO as a result of increased oxidant stress generated by the dialysis process (201, 238, 239). NO is anti-atherogenic and is produced by vascular endothelium in response to physical and chemical stimuli. Damage to the endothelium, an established marker of early atherogenesis, decreases NO production which can be assessed by measurement of endothelial dependent flow mediated blood vessel dilatation using high resolution ultrasound (see chapter 1.5).

There is, however, conflicting evidence regarding the acute effect of haemodialysis on endothelial function; two studies have shown the process to have a beneficial effect (159, 240) and another has shown an adverse effect (201). All these studies were in adults. To date the acute effect of haemodialysis in children has not been investigated. Reasons for the differing results may be that cardiovascular disease was already established in the patients or that the fluid shifts that occurred during dialysis influenced the results.

It is important to establish whether haemodialysis has an adverse effect on the endothelium and, if so, the mechanism whereby this occurs, in order to determine appropriate preventative therapies. Many children continue to produce urine and do not require fluid removal during the haemodialysis process, thereby removing the potential effect of large changes in extravascular fluid volume on blood pressure and endothelial function making results easier to interpret.

Therefore, endothelial function was assessed before and after a session of euvolaemic haemodialysis in a small group of children. In addition to measuring dialysis adequacy changes in oxidant stress and NO metabolites were measured.

6.3 Subjects and methods

6.3.1 Patients

Seven stable children receiving euvolaemic haemodialysis at Great Ormond St Hospital were entered into the study. Exclusion criteria were age <7 years (to ensure cooperation with the study), CRF of inflammatory cause, diabetes, arterio-venous fistulae in both arms, significant arterial/venous stenoses in proximal blood vessels, concurrent medication with immunosuppressants, and those with inter-dialytic fluid gains. The dialyser membranes used were all semi synthetic and biocompatible, Cobe from Gambro USA (haemophane) and Nipro FP 70 from Nipromedical Corporation Japan (triacetate cellulose and diacetate cellulose).

6.3.2 Assessment of conduit artery endothelial function.

Endothelial function was determined by recording the dilator response of the brachial artery to increase blood flow generated during reactive hyperaemia of the downstream forearm flow mediated dilatation (FMD), as discussed in Chapter 3, section 2.

6.3.4 Protocol

Blood pressure and endothelial function was assessed at baseline (predialysis), and at 1 hour, 3 hours and 5 hours post dialysis using the same segment of brachial artery. Blood was drawn predialysis and at 1 hour post dialysis. The subjects' weights pre and post dialysis were recorded.

6.3.5 Biochemical measurements

Blood samples were taken for urea, creatinine, venous bicarbonate, haemoglobin, C-reactive protein (CRP), folic acid, nitrotyrosines, total antioxidant activity of the serum and LDL lag times. Methodology as discussed in Chapter 3.

6.3.6 Statistics

All data are expressed as mean \pm SD and were checked for normal distribution. Group mean values pre-dialysis were compared with group mean values at each individual time point post dialysis using the paired Student t test. Significance was assumed at $p<0.05$.

6.3 Results

6.3.1 Baseline characteristics

Six of the seven children completed all studies and one child elected to omit the 5 hour study. Their clinical characteristics are shown in table 6.1. All studies were performed without significant change in weight pre and post dialysis (Table 6.2)

Age (years)	12±3
Sex F:M	3:4
Aetiology of ESRF	
Congenital dysplasia	6
Congenital nephrotic	1
Access	
Tunneled line	6
AVF	1
Systolic BP (mmHg)	127 ± 19
Diastolic BP (mmHg)	73 ± 10
KT/V	1.46 ± 0.37
URR (%)	71 ± 0.37
Dialyser	
COBE 400	1
COBE 500	5
NIPRO FP70	1

Table 6.1: Patient characteristics

	Baseline	Post	P value
Baseline flow (VTI, m)	0.07±0.02	0.09±0.02	ns
Baseline diameter (mm)	3.26±0.6	3.32±0.6	ns
Peak hyperaemia (%)	444±227	345±237	ns
FMD (%)	7.16±3.6	4.91±2.1	0.067
GTN (%)	10.97±7.4	10.52±4.5	0.755
Systolic BP (mmHg)	126±18	128±22	ns
Diastolic BP (mmHg)	71±9	73±18	ns
Weight (Kg)	46.3±16	45.7±16	ns

Table 6.2: Physical characteristics pre and post dialysis. Expressed as means ± SD.

6.3.2 Biochemistry and haematology results (table 6.3)

As expected, there was a fall in creatinine and urea post dialysis ($p < 0.001$). No significant changes were noted in haemoglobin, serum folate, venous bicarbonate or C-reactive protein. The most striking change produced by haemodialysis was a marked reduction in total antioxidant activity of the serum (252 ± 96 to 33 ± 24 microM trolox Eq $p = 0.001$). There was a small increase in LDL lag times, a marker of LDL resistance to oxidation (21 ± 9 to 24 ± 8 mins $p = 0.02$). There was a tendency for nitrotyrosines to increase following haemodialysis (0.034 ± 0.03 to 0.065 ± 0.05 $p = 0.067$) but this did not reach significance.

	Pre dialysis	Post dialysis	P
Creatinine (nr 40-120 μ mol/L)	754 ± 122	289 ± 97	< 0.001
Urea (nr 4-7 mmol/L)	17 ± 3.4	4.8 ± 1.6	< 0.001
Venous bicarbonate (25-35 mmol/L)	23 ± 3.9	27 ± 1.8	ns
Hb (nr 13-16g/dl)	9.7 ± 1.2	9.4 ± 1.2	ns
Serum folate (2 - 20 μ g/l)	12.7 ± 5.6	13.6 ± 1.5	ns
C-reactive Protein (< 4 mg/L)	2.35 ± 1.8	2.9 ± 1.36	ns
Lag times (n 60mins)	21 ± 9.4	24 ± 8.1	0.02
AOX (n=440 μ M trolox Eq)	252 ± 96	33 ± 24	0.001
Nitrotyrosines (nr 0.02-0.007BSA equivs μ g/mg protein)	0.04 ± 0.03	0.07 ± 0.05	0.067

Table 6.3: Biochemical changes following dialysis expressed as mean \pm SD

6.3.3 Vascular studies (Table 6.4)

In each child arterial diameter, blood pressure and heart rate did not differ significantly at any point in the study from that which was measured at baseline. In addition the stimulus of flow was not significantly different at any point in the study.

	Baseline study	1 hour post	3 hours post	5 hours post (n=6)
Baseline brachial artery blood flow (VTI, m)	0.068±0.02	0.088±0.02	0.096±0.04	0.084±0.03
Systolic BP (mmHg)	126±18	128±22	131±25	122±17
Diastolic BP (mmHg)	71±9	73±18	76±8	73±10
Heart rate (per min)	90±20	98±32	88±19	105±32
Reactive hyperaemia (%)	444±227	345±238	394±215	284±64
FMD (%)	7.16±3.59	4.94±2.07 §	5.07±2.4	5.11±5.14
GTN (%)	10.96±7.41	10.52±4.5 *	9.07±4.46	11.06±8.11

§ p=0.06

* p=0.75

Table 6.4: Vascular responses to haemodialysis in 7 subjects (except where n=6) at all time points. Data are expressed as mean±SD with no significant differences to baseline.

Endothelial dependent dilatation (FMD)

There was a trend for the FMD, and hence endothelial function, to worsen immediately post dialysis (7.16±3.59 to 4.94±2.07 % p=0.06), figures 6.1 and 6.2, without any significant change in the flow stimulus (reactive hyperaemia 444±227 to 345±238% p=ns) or the physical characteristics of the subject (table 6.2). Subsequently the FMD was restored at 3 and 5 hours post dialysis to values which were impaired, but not significantly so, from baseline (p=0.14 and p=0.37 respectively) (figure 6.2).

Endothelial independent dilatation (GTN)

There was no significant change in brachial artery response to GTN at any point compared with baseline (figures 6.1 and 6.2).

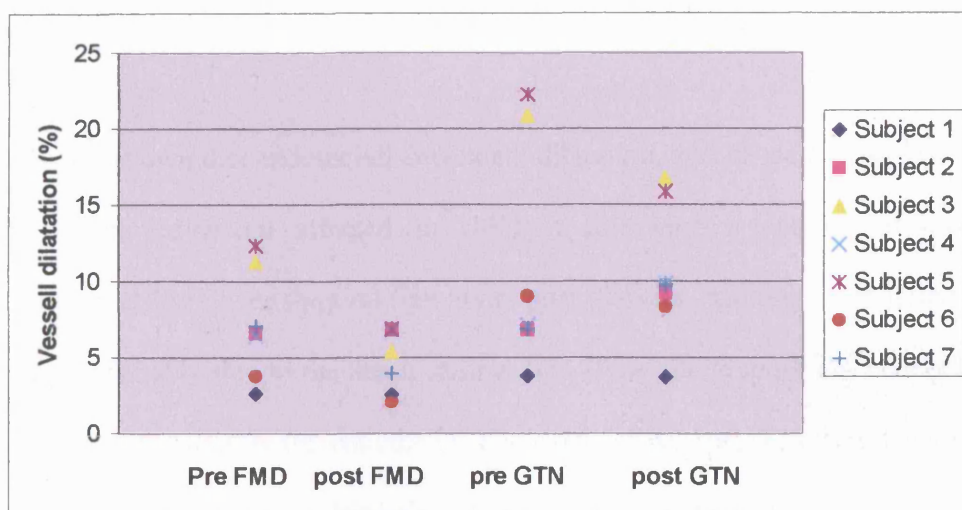


Figure 6.1: Individual vascular responses to haemodialysis.

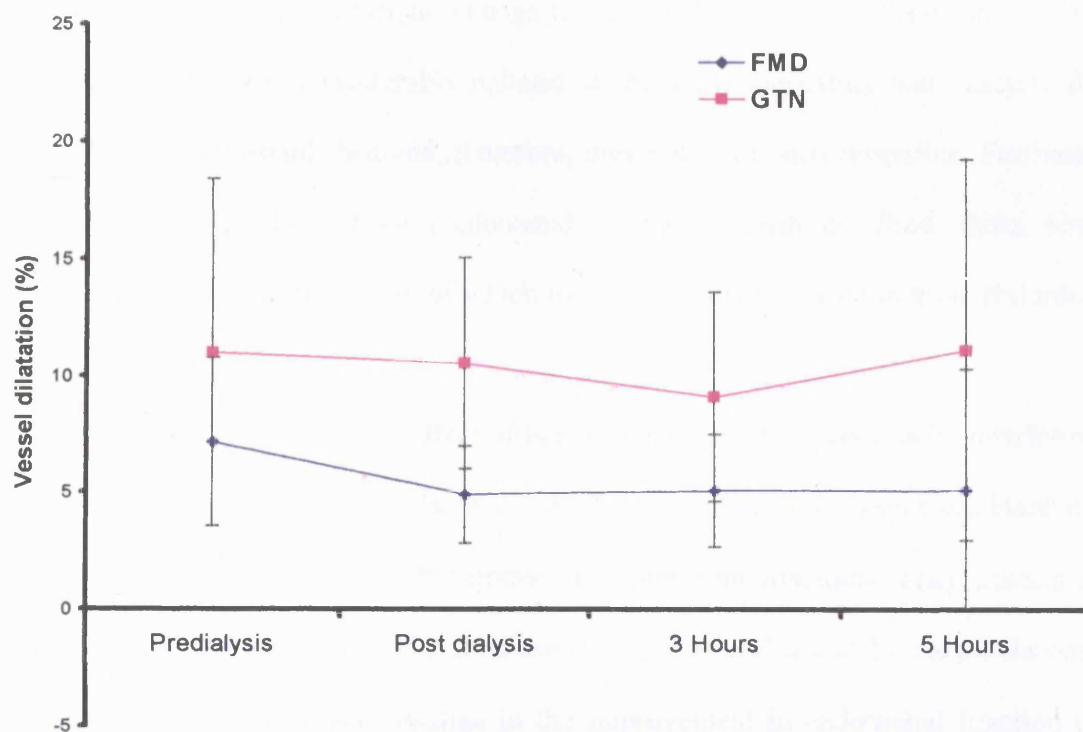


Figure 6.2: Vascular response to haemodialysis (n=7). Data are expressed as group means \pm standard deviation.

6.5 Discussion

This study has shown that endothelial dependent dilatation, and hence endothelial function, is likely to be adversely affected in children following a session of euvolaemic haemodialysis, at least over the first five hours post dialysis. Although this failed to reach significance, probably due to the small sample size. This effect could not be explained by differences in the stimulus for endothelial dependent dilatation (reactive hyperaemia) or altered smooth muscle response (endothelial independent dilatation).

Previous studies in adults have been inconsistent. Two have shown improvements in endothelial function (159,240) after haemodialysis whereas one has demonstrated a deterioration and another no change (201,241). However, in all but one of these studies (241) FMD was considerably reduced at the start, suggesting that vascular disease was already well established and, therefore, may not have been reversible. Furthermore, these studies might have been confounded by factors such as, fluid shifts, smoking and established arteriopathy, all of which we purposefully excluded in these children.

One way that the adverse effect of haemodialysis might operate is by interfering with NO production or metabolism. Here also conflicting results have been seen. Hand et al studied eight adult patients after haemodialysis using venodilatation. They postulated that the removal of inhibitors of NO synthase (L-arginine analogues) by haemodialysis increased NO bioavailability and resulted in the improvement in endothelial function (159). This theory was supported by similar improvements seen in the same patients with the infusion of L-arginine, the precursor of NO. Cross et al in 2001 also found a reduction in L-arginine

analogues after haemodialysis but there was no correlation with FMD. In these studies NO bioavailability appeared increased as a consequence of the haemodialysis process (240).

However, in contrast, the pro-inflammatory effect of haemodialysis acts to increase oxidative stress. This is associated with inhibition of NO synthase and the inactivation of NO. Miyazaki (201) postulated that the deleterious effect of HD on endothelial function in the 12 adults they studied was a result of a pro-inflammatory reaction at the dialyser membrane generating reactive oxidant species, as the effect was reversed by a vitamin E coated dialyser. In addition, there was a significant rise in levels of oxidised LDL (oxLDL, a product of increase oxidative stress) in these patients, which correlated with FMD. This contradictory effect may have been due to the type of dialyser used, which was less biocompatible than in previous studies. This is supported by recent work from Kosch et al in adults who showed that only the less biocompatible cellulose dialyser membranes produced an adverse effect on endothelial function (FMD 9.4 ± 2.1 to 7.4 ± 1.8 % p 0.05) with no change seen using synthetic, biocompatible membranes (242).

However, despite the use of biocompatible membranes, I still found a highly significant reduction in total antioxidant activity of the serum and an increase in nitrotyrosines (products of increased oxidative stress) associated with the blunted FMD response. This suggests that antioxidants were removed and/or consumption of antioxidants was increased. This is in keeping with other studies that have shown elevated products of oxidative stress such as plasma oxLDL, lipid peroxides and reductions in serum antioxidants following a single session of haemodialysis (243, 244, 245, 246), in addition to a correlation between duration of dialysis and markers of oxidant stress (246). These

effects are brought about at the dialysis membrane by the activation of polymorphs with the generation of reactive oxygen species and triggering of the inflammatory response (237). In this study there was no increase in CRP levels. This suggests that either the rise is delayed (ie blood samples taken too soon) or that the direct generation of reactive oxygen species, rather than inflammation was the main cause for the reduction in total antioxidant activity of the serum.

Although very low baseline values (compared with previously published data) of lag times of LDL were found, there was a significant but small increase following haemodialysis; i.e. LDL became more resistant to oxidation. LDL is particularly susceptible to oxidation (due to a high concentration of polyunsaturated fatty acids) and increases in levels of oxidized LDL have been demonstrated following a single session HD (243). Oxidised LDL is implicated in the formation of atherosclerotic plaques. LDL resists oxidation by virtue of lipid soluble antioxidants within the macromolecule, and it may be that in this study, haemodialysis resulted in acute concentration of lipid soluble antioxidants. On any count both pre and post dialysis lag times are both very low indicating a significant oxidant stress as the dominating phenomenon. This would account for the rise in oxidised LDL levels seen in other studies. Unfortunately we did not measure oxidised LDL levels.

Overall, the net effect of haemodialysis in this study would appear to be one of reduced NO bioavailability and endothelial dysfunction.

With the ever-increasing population of paediatric patients on haemodialysis these findings are of clinical relevance. If the current methods of haemodialysis exacerbate the adverse effects of renal failure on vascular biology, further research is required such that these

effects could be prevented or retarded, thereby resulting in a subsequent reduction in cardiovascular mortality and morbidity.

Chapter Seven

Summary

7.1 Main Observations

7.1.1 L-arginine Study

Results showed that oral supplementation with L-arginine for 4 weeks did not improve brachial artery endothelial function, despite achieving plasma levels of twice normal. It also demonstrated that oral L-arginine was unlikely to be a useful therapeutic tool in the form used due to the unpleasant taste and the adverse biochemical profile it produced. It would have been difficult to identify any direct positive benefit on NO bioavailability in the face of the increased oxidant stress produced by its use.

7.1.2 Folic acid Study

This study showed that supplementation with high dose folic acid for eight weeks resulted in reduction in homocysteine levels, decrease in LDL susceptibility to oxidation and improvement in endothelial function.

However the results of this study should be interpreted with caution as the final FMD after placebo and active phases were not significantly different. The benefit seen perhaps because the pre treatment FMDs were different. Although no carry over effect was seen for homocysteine levels it is difficult to ignore the carry over effect seen for folic acid levels. The washout period was probably too short.

7.1.3 Haemodialysis Study

This was a small study which showed that there was a trend for endothelial function to be adversely affected in children following a session of euvolaemic haemodialysis, at least over the first five hours post dialysis. This did not reach significance. However the fall in total antioxidant activity with a single session of haemodialysis did reach significance suggesting that the process of haemodialysis produces oxidant stress.

7.2 Future work

The principle of L-arginine supplementation is sound but its effect on the urea cycle makes it a useless therapeutic tool in renal failure in the chronic setting. The benefits of oral supplementation seen in both animal and human studies in high risk groups have not been conferred on the CRF population in either adults or children. Further clinical studies are probably not warranted.

More studies of folic acid supplementation are necessary, particularly long term to see if the benefit on endothelial function it produces improves cardiovascular outcome. There is a relative resistance to folic acid in CRF, which could perhaps be overcome by the addition of co-factors or active precursors.

The haemodialysis study should be extended, as certainly there was a trend for haemodialysis to result in a blunted FMD response. The sample size was however too small for the result to reach significance.

If subsequent studies showed haemodialysis to have a significant adverse effect, more studies of the mechanisms and interventional studies should follow.

Clinical intervention studies not yet tried may be the use of statins, vitamins E and C and perhaps ACE inhibitors. Calcium phosphate metabolism is a key area.

The non-invasive technique of FMD lends itself well to the investigation of vascular pathology in children even in large-scale trials. The association between endothelial dysfunction and subsequent risk of cardiovascular mortality has been demonstrated prospectively in adults but not children. A long-term, large-scale, observational study of endothelial dysfunction associated with childhood onset CRF patients and its relationship to subsequent cardiovascular morbidity and mortality would be useful. If endothelial dysfunction, using FMD is confirmed as an independent predictive risk factor in children it could be used as a clinical tool.

If, as already suggested by epidemiological data, the paediatric population has relatively worse cardiovascular outcomes than adults, it is essential to pursue the interventional studies. If therapeutic intervention is available it could be used in conjunction with FMD before cardiovascular disease is apparent. The clinical implications are highly significant.

7.3 Conclusion

The increase in size of the stock of the paediatric renal patients on RRT by 37% in 10 years is of increasing concern. If mortality rates from cardiovascular disease for adults with CRF and RRT, from cardiovascular disease, far exceed that of the general population, what is the future for the paediatric population?

Children with CRF and on RRT already have evidence of vascular disease and will be exposed to many more years of adverse chemistry making it likely that we will see problems occurring at an earlier age. Having survived their childhood years, death from cardiovascular disease in early adulthood may become inevitable.

Early intervention is the key, and this thesis concerns the development of primary prevention at a time when either adverse vascular biology could be prevented or reversed. This differentiates it from adult studies in which a high number of subjects have established vascular disease. Clinical intervention studies examining endothelial function in children with CRF, to my knowledge and at the time of writing had not previously been performed.

It is clear that factors other than those traditionally recognised, such as diabetes, hypertension, hypercholesterolaemia, and smoking are associated with the development of atherosclerotic disease in renal failure. If paediatric patients are to expect a reasonable prospect, care should also be taken in the management and investigation of the contributory factors discussed in this thesis.

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